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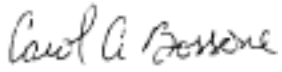

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ABSTRACT

Title of Dissertation: Changes in inhibitory control of circular smooth muscle during colitis in the rat.

Carol Ann Bossone, DVM, 1998

Dissertation directed by: Terez Shea-Donahue, Ph.D., Research Associate Professor of Medicine and Physiology.

Colitis causes symptoms of diarrhea interspersed with periods of quiescence. Changes in smooth muscle contractility may contribute to abnormal motility seen in inflammatory bowel disease. Inhibitory neurotransmitters contribute a significant role in maintaining normal motility and may be altered during inflammation. The aim of this study was to compare inhibitory mechanisms of normal (CONTROL) with inflamed animals. Male Sprague-Dawley rats received intrarectal saline (CONTROL) or trinitrobenzenesulfonic acid in 50% ethanol. The distal colon was removed after 4 hours (ACUTE) or 28 days + 4 hours after saline (HEALED) or trinitrobenzenesulfonic acid (REINFLAMED). In circular smooth muscle, concentration-dependent responses to potassium and acetylcholine as well as to substance P and neurokinin A were determined in the presence of antagonists (atropine, N^G-nitro-L-arginine,aminoguanidine, apamin, tetrodotoxin, and hexamethonium). Spontaneous contractions were determined in the presence of nitric oxide synthase inhibitors, N^G-nitro-L-arginineand aminoguanidine. The amplitude of spontaneous contractions was significantly ($p < 0.05$) increased from

CONTROL (1529 ± 337 mN/cm^2) in ACUTE (6755 ± 1004 mN/cm^2) and REINFLAMED (6705 ± 2526 mN/cm^2), while frequency was increased from CONTROL (9 ± 2 contractions/l0 seconds) in HEALED (27 ± 4 contractions/l0 seconds) and REINFLAMED (27 ± 6 contractions/l0 seconds). Responses to acetylcholine, substance P, and neurokinin A in HEALED and REINFLAMED were not significantly different from CONTROL. Responses to tachykinins were similar also in the presence of hexamethonium, N^G-nitro-L-arginine + apamin, and N^G-nitro-L-arginine + atropine. In contrast, acute inflammation significantly increased ($p< 0.05$) the response to substance P and acetylcholine, but decreased ($p < 0.05$) the response to neurokinin A. However, no change from vehicle treated tissue was noted for substance P in the presence of atropine, hexamethonium, or N^G-nitro-L-arginine. Aminoguanidine and tetrodotoxin increased responses to both tachykinins in all groups. The reduction of muscarinic, ganglionic, and nitrergic control in ACUTE returned in HEALED and REINFLAMED, but not without alterations in inhibitory control. Differences between ACUTE and REINFLAMED reflect remodeling of cholinergic, nitrergic, and ganglionic control of smooth muscle and a change in the source of nitric oxide that occured during inflammation. This may contribute to the excitable motility patterns seen during the initial and relapse stages of colitis.

CHANGES IN INHIBITORY CONTROL OF CIRCULAR SMOOTH MUSCLE DURING COLITIS IN THE RAT

by

Carol Ann Bossone

Dissertation submitted to the Faculty of the
Department of Physiology Graduate Program of the
Uniformed Services University of the Health
Sciences in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy 1998

DEDICATION

This thesis is dedicated to all God's creatures in particular laboratory animals. It is for these often unrecognized species that this paper as well as research itself has progressed as far as it has today and for the future to come. These animals have contributed greatly to the knowledge we have gained to benefit all living organisms. Research is made possible by these animals which have profoundly contributed to the betterment of human existence. Their contribution to humanity reaches us through our families (in particular my own parents and sisters), friends, and even our companions (pets). We owe them our greatest respect and gratitude for we are all God's creatures. As one famous veterinarian wrote very eloquently, we truly are...

All creatures great and small

All things bright and beautiful

All things wise and wonderful

The Lord God made them all

James Herriot

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To my thesis committee, Dr. James Terris, Dr. Sheila Muldoon, Dr. David Livengood, Dr. David Dobbins, I am truly grateful to them for their undying commitment, and advise on scientific matters. Their insights have been invaluable to me and will be remembered throughout my career.

A special thanks goes to my advisor, Dr. Terez Shea-Donohue who is my best friend, teacher and mentor. Her “remarkable” talent and “fine” character “ques” nothing short of brilliance. Through my short tour in her laboratory I’ve learned a tremendous amount about GI physiology as well as so many other “remarkable” things. I am indebted to her many times over for this “icebreaking” research and it is because of her I can finally say “We did it!“.

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LIST OF ABBREVIATIONS

ACH	Acetylcholine
AG	Aminoguanidine (selective iNOS inhibitor)
ATP	Adenosine triphosphate
cAMP	3' 5' cyclic adenosine monophosphate
cGMP	3' 5' cyclic guanosine monophosphate
EC_{50}	Concentration at which 50% of the maximum response occurs
ENS	Enteric Nervous System
GI	Gastrointestinal
GMC	Giant Migrating Complex (contraction)
HEX	Hexamethonium (ganglionic blocking agent)
IBD	Inflammatory Bowel Disease
ICC	Interstitial Cells of Cajal
KC1	Potassium chloride
L-NNA	N^g nitro-L-arginine (NOS inhibitor)
L_o	Optimum length
LTB_4 LTC_4 LTD_4	Leukotriene B4, Leukotriene C4 Leukotriene D4
M	Muscarinic (receptor)
mN	milli-Newton
N	Nicotinic (receptor)
NANC	Non adrenergic non cholinergic

NKl	Neurokinin 1 (receptor)
NK2	Neurokinin 2 (receptor)
NKA	Neurokiuin A
NKB	Neurokinin B
NO	Nitric oxide
NOS	Nitric oxide synthase
cNOS	Constitutive nitric oxide synthase
eNOS	Endothelial nitric oxide synthase
iNOS	Inducible nitric oxide synthase
nNOS	Neural nitric oxide synthase
PGE	Prostaglandin E
$PGF_{2\ alpha}$	Prostaglandin $F_{2\ alpha}$
PMN	Polymorphonuclear cells
SP	Substance P
TNBS	Trinitrobenzenesulhonic acid
TTX	Tetrodotoxin
VIP	Vasoactive intestinal peptide

INTRODUCTION

ENTERIC NERVOUS SYSTEM

Colonic smooth muscle contractility depends upon the net influence of the central nervous system, extrinsic autonomic nervous system and the enteric nervous system as well as unique, inherent properties of the gut smooth muscle. The enteric nervous system locally controls the ability of smooth muscle to contract and functions as the first level of intrinsic control in the regulation of motility and secretion. The autonomic and central nervous system serve as higher levels of extrinsic control and are important in the more integrated reflexive function of motility and secretion (Figure 1). Within the enteric nervous system are two plexuses, the submucosal complex (beneath the surface epithelial layer) and the myenteric plexus (between circular and longitudinal muscle layers). This latter plexus is important for much of the normal motor function of the intestine, while the submucosal complex is more important in the secretory and vasomotor function (Dockray, 1987).

The enteric nervous system receives input from the autonomic nervous system by parasympathetic nerves acting on preganglionic nicotinic receptors and by sympathetic nerves acting on postganglionic neurons.The parasympathetic system has an excitatory influence while the sympathetic nervous system is primarily inhibitory. Much of the extrinsic innervation is involved in both inhibitory and excitatory reflexes involved in long distance communication throughout the gastrointestinal tract. Acetylcholine (Ach)

is the preganglionic neurotransmitter at nicotinic receptors and is also the excitatory neurotransmitter at postganglionic nerve terminals via muscarinic receptors. Ach can also act as a preganglionic transmitter at noncholinergic-nonadrenergic (NANC) neurons which then releases a variety of neuropeptides (Galligan, 1993; Goyal and Hirano, 1996). In addition, there are also intrinsic sensory neurons from both muscle and mucosal receptors transmitting information to the enteric nervous system. Finally, there are inter-neurons that integrate and modulate information within the enteric nervous system (Figure 1). These coordinated activities, in conjunction with the inherent properties of intestinal smooth muscle, are responsible for the motility and digestive processes that occur throughout the gastrointestinal tract (Dockray, 1987; Goyal and Hirano, 1996).

SMOOTH MUSCLE PROPERTIES

Smooth muscle contraction in the GI tract is dependent on properties inherent in this muscle that allow it to perform specific functions of motility. Smooth muscle, like skeletal muscle, relies on a source of intracellular Ca^{+2} for the actin and myosin interaction and ultimate contraction. Smooth muscle, unlike skeletal muscle, does not have large stores of intracellular [Ca^{+2}], (Grider and Maklouf, 1988). Ca^{+2} influx is significantly important and can be initiated by voltage or receptor mediated Ca^{+2} channels or by a depolarizing stimuli such as K^{+}. Intracellular calcium's interaction with the actin and myosin produces a contraction. When optimized by stretch or length increases, smooth muscle can generate a maximum contractile force in response to a depolarizing agent. The length of the muscle at which maximum tension is developed is called the optimum length (L_O). The tension that develops in response to the depolarizing stimulus

is defined as active tension, while passive tension is that tension generated by the tissue prior to the contractile response. Total tension is the sum of active and passive tensions.

Oscillations of the smooth muscle membrane potential control the time and direction of contractions. Due to changes in ionic conductances, the membrane potential rhythmically fluctuates. When these periodic depolarizations, called basal electrical rhythm, reach threshold the muscle generates an action potential and the muscle contracts. Specialized cells within the circular smooth muscle called interstitial cells of Cajal (ICC) are responsible for generating the basal electrical rhythm and function as pacemaker cells for the colon (Rumessen and Thuneberg, 1996; Sarna, 1991). Action potentials are generated in response to neural and/or chemical signals such as neurotransmitters. If the neurotransmitter is excitatory (Ach) the depolarization will exceed threshold and a contraction will occur. Conversely if an inhibitory neurotransmitter is released, threshold will not be reached and no contraction will occur (Dockray, 1987). The spontaneous contractions generated by the smooth muscle represent the final result of these pacemaker cells.

These electrical events result in three types of mechanical contractions: tonic, special, or phasic. Tonic contractions are important in regions such as sphincters and function to minimize reflux passage of material. Special contractions refer to motility patterns specific to a particular region of the GI tract such as mixing movements in the intestines and haustral migrations, and mass movements in the colon. These latter patterns are important for the storage and elimination functions of the colon. In the colon, the phasic contractions are controlled by myogenic, neural, or chemical mechanisms. These contractions are poorly coordinated in space and tend to propagate with a greater

strength of contraction but over shorter distances than that seen in the small intestines. The pattern of circular muscle contractions in the colon can be altered by the pacemaker cells. These can change the generation and/or pattern of spontaneous contractions in the colonic smooth muscle, and depending on the neurotransmitter (or lack of) released, can alter the motility pattern (Rumessen and Thuneberg, 1996).

FIGURE 1

Enteric Nervous System

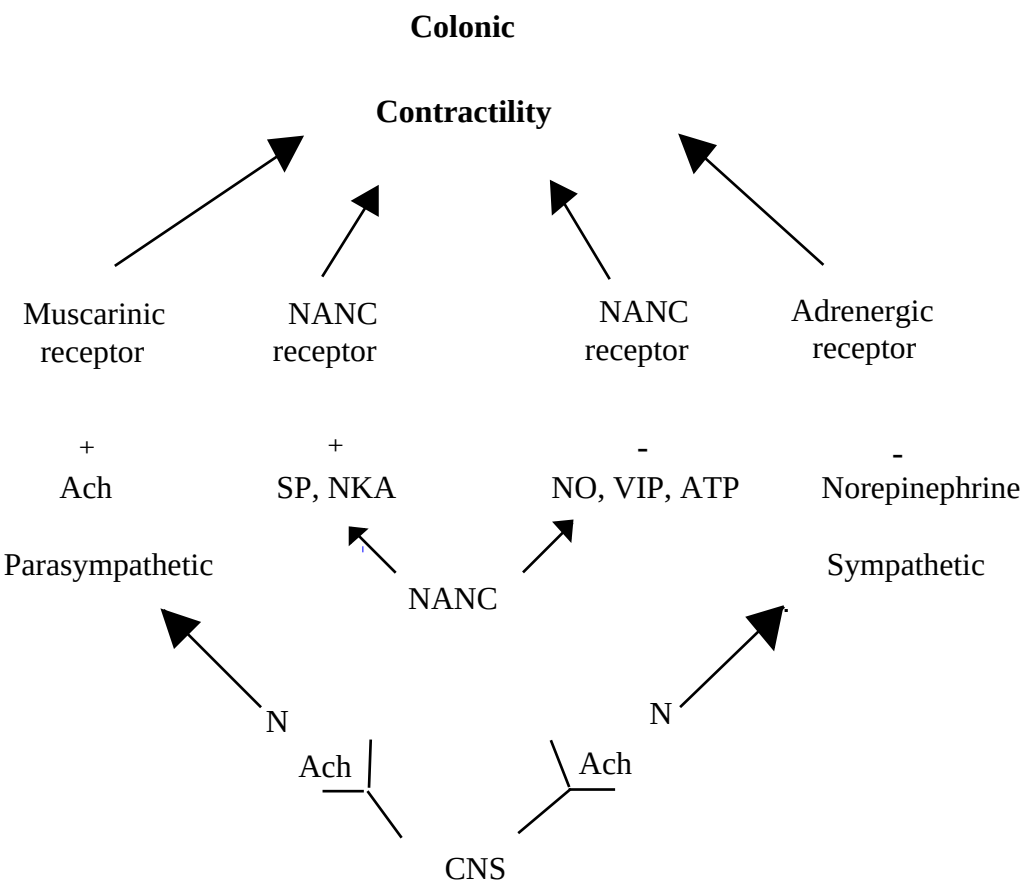


Figure 1. Enteric Nervous System and regulation of colonic contractility

Ach is a major transmitter of the excitatory motor neurons for both nicotinic and muscarinic receptors in the enteric nervous system. In the colon, nicotinic receptors are present on the ganglionic neurons of the parasympathetic nervous system that are located in the myenteric plexus. Muscarinic receptors are present on postganghonic neurons and on smooth muscle. There are three major muscarinic receptors; Ml on postsynaptic nerve terminals; M2 on presynaptic nerve terminals; and M3 on glandular tissue and smooth muscle (Dockray, 1987; Felder, 1995). Atropine, a competitive muscarinic antagonist, effectively blocks all three types of M receptors. Nicotinic receptors can be effectively blocked by the actions of hexamethonium, a nondepolarizing ganglionic antagonist.

Neurotransmitters at the level of the postganglionic fibers of the parasympathetic nervous system can be Ach or a group of mediators collectively called nonadrenergic noncholinergic neurotransmitters (NANC). These neurotransmitters can be excitatory or inhibitory and are characterized by their pharmacological similarities. The tachykinins substance P (SP) and neurokinin A (NKA) and neurokinin B (NKB) are peptides that share similar pharmacological properties and are important excitatory neurotransmitters. They are classified by their affinity to receptors with SP binding preferentially to NK-1 receptors, NKA to NK-2 and NKB to NK-3 receptors respectively . NK-1 receptors appear on both nerve and muscle of the enteric nervous system, while NK-2 receptors are primarily on muscle and NK-3 are on nerves. Of these three tachykinins, SP and NKA appear to be more important in the colon as NKB is absent in peripheral tissue (Hellstrom, et al., 1994; Galligan, 1993; Tsukamoto, et al., 1997). SP is by far the most

intensively studied tachykinin with most recent studies suggesting its role as an excitatory neurotransmitter acting within the enteric nervous as a sensory neuropeptide, and directly on the intestinal smooth muscle (Dockray, 1987; Fontaine and Lebrun, 1989; Koelbel, et al., 1989). NKA in contrast, has not been as well characterized, but appears to act primarily on the smooth muscle (Galligan, 1993). Both tachykinins act to depolarize smooth muscle resulting in an increase in membrane input resistance which ultimately cause the release of intracellular Ca^{+2} and contraction (Grider, 1993; Galligan, 1993).

In addition to the excitatory neurotransmitters there are also enteric inhibitory neurotransmitters. The inhibitory innervation of the gut is of great significance to the overall motility pattern of the colon. Smooth muscle is normally under tonic suppression by myenteric neurons that stimulate the release of NANC inhibitory neurotransmitters. Thus the overall function of the enteric nervous system is to regulate and control the myogenic excitability of smooth muscle. Most of these conclusions were based on studies in which this inhibition was removed by the nerve blocking agent tetrodotoxin. The resulting myogenic excitation demonstrated that a neurogenic component was involved in inhibitory innervation of the gut (Wood, 1975).

Some of the neurotransmitters ultimately responsible for the inhibition of colonic smooth muscle contraction include ATP, neuropeptides such as vasoactive intestinal peptide (VIP), and opioid peptides, galanin, and nitric oxide (NO). Of these inhibitory neurotransmitters probably the most studied and more important ones are NO, ATP, and VIP. NO has been identified as an important inhibitory neurotransmitter or neuromodulater in the enteric nervous system (Delbro, 1996; Hata, et al., 1990; Iverson, et al., 1994; Keef, et al., 1993; Makhlouf and Grider, 1993; Ward, et al., 1992).

Numerous studies have been conducted to show the existance and/or coexistance of these neurotransmitters within the enteric nervous system and colon specifically (Burnstock, 1994; Domoto, et al., 1995; Nichols, et al., 1994; Shuttleworth, et al., 1993). These neurotransmitters all cause relaxation of smooth muscle, however, their mechanisms of action differ. VIP and ATP act to increase cAMP altering K conductance to cause hyperpolarization (Laburthe, et al., 1993). The actions of these neurotransmitters can be almost entirely blocked by apamin, a K^+ channel blocker.

NO (and citrulline) in contrast, is synthesized from its precursor L-arginine by the enzyme nitric oxide synthase (NOS). The NO generated stimulates cGMP secondary to activation of soluble guanylate cyclase causing relaxation of smooth muscle. NOS containing neurons have been found in the myenteric plexus throughout the gastrointestinal tract (Nichols, et al., 1994; Matini, et al., 1995; Shuttleworth, et al., 1993). NOS exists in three isoforms (Knowles and Moncada, 1994; Marletta and Maxey, 1995). Endothelial NOS (cNOS), found in vascular endothelial cells, and neuronal NOS (nNOS) are Ca^{+2}-calmodulin dependent. These two are broadly defined together as constituitive (cNOS). Finally, there is an inducible NOS (iNOS) found in certain inflammatory cells. Based on studies utilizing competitive inhibitors, cNOS has been found to be responsible for the basal release of NO from neuronal and/or endothelial type cells. In addition, enteric neurons in the proximal colon can also recycle citrulline to arginine to sustain nitrergic neurotransmission (Shuttleworth, et al., 1995). In contrast, iNOS is not normally present in these cells, but is induced under conditions in which cells, such as macrophages, are activated. Inducible NOS produces large amounts of NO from L-arginine in these cells for long periods of time when “induced” by endotoxin and

cytokines (Marletta, et al., 1988; Hibbs, et al., 1987; Lefer and Lefer, 1993; Moncada, et al., 1991).

Since the initial observations of NO there have been a large number of arginine analogs that have been developed that inhibit NO production and have significantly furthered the understanding of the role of NO in the gastrointestinal tract (Moncada, et al., 199 1; Knowles and Moncada, 1994; Lefer, and Lefer, 1993). Competitive inhibitors of the NOS enzyme include Ng nitro-L-arginine (L-NNA), Ng monomethyl L-arginine (L-NMMA), and Ng nitro-L-arginine methyl ester (L-NAME). L-NNA is a potent inhibitor of constitutive NOS, that has also been shown to inhibit iNOS synthesis (Joly, et al., 1994). Aminoguanidine (AG) is an isoform selective antagonist of iNOS. Although AG preferentially inhibits this isoform, recent evidence has shown that AG at high concentrations can also inhibit the cNOS isoform (Wolff and Lubeskie, 1995). Because if aminoguanidine's high affinity for the iNOS isoform, it has been used in many studies involving NO (Ribbons, et al., 1995; Lefer and Lefer, 1993). These studies have demonstrated the role of NO in the colon under normal conditions implicating its importance in the regulation of gut function. Many of these investigators have suggested that it may also have a role as a critical mediator during inflammation.

INFLAMMATION

Idiopathic inflammatory bowel disease (IBD) includes a group of disorders with similar clinical and pathological signs. Characterization of these disorders can be difficult, but is often grouped by location and/or pathophysiology. Crohn's disease is described as a chronic transmural inflammatory disease most commonly present in the

ileum and colon, whereas ulcerative colitis is usually limited to the colonic mucosa. Both ulcerative colitis and Crohn's disease progress from an acute to a chronic stage of inflammation which can be distinguished histologically by the types of inflammatory cells present (Yamada, et al., 1992). Generally, chronicity signifies a greater involvement of the lamina propria. IBD is further characterized by periods of quiescence during which spontaneous relapse or acute reinflammation may occur. This stage of the disease is arguably the most important clinically but has not been investigated in an animal model. Early in the disease, the mucosa may be indistinguishable from healthy tissues. The disease is exacerbated during the cycles of remission and relapse and in more advanced disease, the mucosa may be atrophic with a persistent diffuse inflammation. In humans, IBD is usually associated with chronic diarrhea, weight loss, and lower abdominal pain. Chronic colitis is often interfaced with acute bouts of violent diarrhea, fevers, and possible peritonitis. Blood and mucus are often seen in the stool during this period (Berkow, 1994).

A major characteristic of idiopathic inflammatory bowel disease (IBD) is uncontrolled inflammation which is thought to be secondary to activation of the immune system (Rachmilewitz, et al., 1989). Active IBD is associated with a wide spectrum of inflammatory events including increases in neutrophilic infiltration, oxygen radicals, cytokines, and eicosanoids (Allgayer, et al., 1989; Lauritsen, et al., 1986; Rachmilewitz, et al., 1989). Many products may directly or indirectly affect smooth muscle contractility, including eicosanoids (Morteau, et al., 1993; Pons et al., 1992; Sjogren, et al., 1994), tachykinins (Grossi, et al., 1993), and nitric oxide (Sanders, and Ward, 1992). Each of these elements has been proposed to play a role in the secondary amplification of

the inflammatory response which underlies many of the functional changes associated with IBD.

There is little information on the changes in function specific for acute versus chronic inflammation. In addition, the definition of acute versus chronic stages of inflammation is often unclear or inconsistent, even in established animal models of IBD. Previous studies in our laboratory and others, however, indicate that inflammation induces stereotypic alterations in the response of colonic smooth muscle (Hosseini, 1996; Grossi and Collins, 1993). These results suggest that inflammation results in a cascade of events that lead to reproducible changes in gut function that are characteristic of the disease stage. Changes in smooth muscle function upon relapse have not been investigated but would provide insight into a critical component of IBD. Characterization of the response to reinflammation adds an important dimension to a comprehensive study of IBD and may be of use in designing therapies to treat IBD and its recurrence.

Research into the pathogenesis of IBD has made use of several established animal models that utilize different inducing agents. One in particular is the hapten 2,4,6-trinitrobenzene sulfonic acid in ethanol (TNB/ethanol) (Morris, et al., 1989; Conner and Grisham, 1996). This agent produces an acute inflammation which progresses over several weeks to a chronic stage morphologically similar to Crohn's disease. It is a recognized model of inflammation in the rat colon (Morris, et al., 1989; Wallace and Keenan, 1990; Yamada, et al., 1992; Yamada, et al., 1993). Typically, mucosal permeability is increased within 2 hours after exposure to TNBS/ethanol and correlates well with the appearance of mucosal damage (Yamada, et al., 1992). In the rat colon, hyperemia, epithelial sloughing, increased mucosal permeability, enhanced

myeloperoxidase activity, polymorphonuclear infiltration and edema are evident 2 to 6 hours after TNBS/ethanol administration (Yamada, et al., 1992). In the rabbit ileum, mucosal necrosis, neutrophil infiltration, submucosal edema, and vascular changes such as endothelial swelling, leukocyte margination and translocation are observed at 6 hours (Sjogren, et al., 1994; Goldhill, et al, 1995).

The acute stage is followed by a chronic inflammation that is evident at 48 hours (Yamada, et al., 1992) and progresses for several weeks (Morris, et al., 1989; Yamada. et al., 1992). In the colon, this stage is marked by the presence of mononuclear cells such as lymphocytes and macrophages increased colonic weight (Yamada, et al., 1992a), angiogenesis, hypertrophy of the muscularis mucosae (Allgayer, et al., 1989), adhesions, and tibroplasia (Rachmilewitz, et al., 1989). In both acute and chronic inflammation, alterations in gastrointestinal motility leading to diarrhea are a frequent complication (Sethi and Sarna, 1991). Moreover, diarrhea is often the first symptom upon disease relapse.

Previous data have shown that intraluminal exposure of rabbit ileum to two different agents, TNBS/ethanol or the cytotoxic lectin, ricin, induced similar changes in histological damage and eicosanoid production (Sjogren, et al., 1994). Although there was no injury to the muscle layers in this acute model, myoelectric activity measured as amplitude, frequency and direction, were significantly increased. Subsequent *in vitro* studies in isolated circular smooth muscle strips taken from inflamed ileum exhibited alterations in both excitatory and inhibitory neurotransmission which may account for the altered motility observed *in vivo* (Goldhill, et al., 1995).

In the normal colon, inhibitory neurotransmission plays an important role in

controlling the inherently excitable smooth muscle contractility. There is evidence suggesting that alterations in cholinergic and/or tachykinin neurotransmission contribute to the abnormal increase in motility associated with IBD possibly by altering the normal inhibitory control on the colon (Tsukamoto, et al., 1997; Hosseini, 1996). Many neurotransmitters can influence motility by their effects on enteric nerves and by a direct action on smooth muscle. Increased contractions to carbachol were demonstrated in ileal circular smooth muscle obtained from Crohn's patients (Vermillion, et al., 1993). These responses were insensitive to the neurotoxin, tetrodotoxin, and were intensified by atropine, indicating a direct effect on smooth muscle, suggesting that inhibitory control may be acting locally at the muscle.

There are few studies assessing inflammation-induced changes in colonic contractility *in vitro.* Previous work in our laboratory showed that acute colitis evoked an increase in the response to acetylcholine, and substance P, but a decrease in the response to neurokinin A (Hosseini, 1996). The inflammation-induced changes in the response to contractile agents may be the result of altered release or sensitivity to these transmitters. Additionally, there is little if any work on *in vitro* studies of more chronic inflammation.

Chronic colitis appears to produce a general reduction in the smooth muscle response to excitatory stimuli (Grossi, et al., 1993; Hosseini, 1996). In a formalin-immune complex model of colitis in rabbits, decreased contractility of colonic circular smooth muscle to nerve stimulation, substance P, and K^+ depolarization were observed (Percy, et al., 1991). However, there is no information relevant to an experimental model that includes acute reinflammation of a previously healed area.

Recently, the role of nitric oxide inhibitory NANC neurons in acute and chronic

inflammation has been recognized (Boeckxstaens, et al. 1993; Knowles and Moncada, 1994; Miller, et al., 1993). During inflammation NO has effects on motility (Boeckxstaens, et al., 1993; Miller, et al., 1993; Calignano, et al., 1992), mucosal integrity (Hutchison et al., 1990), and possibly secretion and absorption (Gagineila, et al., 1994). In chronic ileitis or colitis the production of NO was found to be elevated (Miller, et al., 1993; Ribbons, et al., 1995; Yamada et al. 1993; Moncada, et al., 1991; Keef, et al., 1993). Miller, et al. (1993) demonstrated that the increase in nitrite, a stable end product of NO, paralleled the changes in myeloperoxidase activity. NO is known to be bacteriocidal and to inhibit lymphocyte proliferation possibly by enhancing macrophagic cytotoxicity (Yamada, et al., 1993b; Knowles and Moncada, 1994; Marletta and Maxey, 1995). Several studies have shown that when macrophages are stimulated, NOS activity is increased (Moncada, et al., 1991; Keef, et al., 1993), leading to the conclusion that an inducible NOS in macrophages may contribute to the inflammatory reaction (Moncada, et al., 1991; Keef, et al., 1993a; Knowles and Moncada, 1994). These investigators and others (Yamada. et al., 1993) suggested that the accumulation of nitrites and release of excess NO may be implicated in mucosal injury and motility disorders.

The importance of inflammation-induced changes in smooth muscle responses to tachykinins and cholinergics is further enhanced by evidence that tachykinins both modulate and are modulated by acetylcholine release and possibly NO . The relationship between acetylcholine and substance P has been described as either synergistic or inhibitory with possible coexistence of the two neurotransmitters in the same neurons (Domoto, et al., 1983). The interaction between NKA and acetylcholine is unclear and the contribution of a cholinergic or nitrergic modulation of tachykinins to the abnormal

smooth muscle response during inflammation is unknown but likely contributes to the abnormal motility associated with IBD.

Previous studies in this laboratory have focused mainly on smooth muscle responses during acute and chronic (7 day) TNBS induced inflammation in the rat colon. These experiments were designed to investigate differences in colonic smooth muscle response to Ach and Kc1 and the tachykinins substance P and NKA, and in control and acute inflammation (Hosseini, 1996). Histologically, acute inflammation resulted in significantly increased vasocongestion of the submucosa and mucosa, and predominantly neutrophilic infiltrations throughout the damaged tissue (Hosseini, 1996). In response to acute inflammation, colonic smooth muscle displayed increased sensitivity to K depolarization, an index of non-receptor mediated contractions, as shown by lowered EC_{50} values (effective concentration at which 50% of the maximum response occurs). Ach and substance P responses were increased in acute tissue, while NKA responses were decreased. In addition, EC_{50} values in acute were comparable to tissue taken from control animals indicating there was no change in sensitivity. In the presence of hexamethonium, Ach showed no change in response to Ach when compared to an increase seen in control tissue. This suggested that a nicotinic mediated inhibition present in control tissue was lost in acute tissue.

Chronically (7 day) inflamed tissue histologically showed more thickening of smooth muscle layers and increase in wet muscle weight, changes not seen in acute tissue. Smooth muscle decreased maximum tension, but not its sensitivity to K. Reductions in maximal responses to NKA, SP, and Ach suggested changes in receptor activity (Hosseini, 1996).

Based on this study acute tissue was found to increase sensitivity to depolarization stimuli. In addition, there was a suggested loss of inhibitory neural control. These studies suggested several probable mechanisms for explaining the loss of neurally mediated inhibition in acute tissue, including loss of an inhibitory mechanism. These studies however, did not investigate the role of nitric oxide as an inhibitory neurotransmitter during inflammation. Since NO has been shown to be a major inhibitory neurotransmitter in the normal colon, determination of its role during inflammation may help explain many of the motility changes seen.

The previous work performed in this laboratory also showed that chronic inflammation (7 day) decreased the response of colonic smooth muscle to SP, NKA, and Ach, in addition to showing more profound physical changes upon histological examination. Unfortunately, the literature contains very little work relevant to the latter stages of inflammation, particularly in the area of smooth muscle contractility changes. In addition, although chronic inflammation (7 days) was looked at (Hosseini, 1996), neither a quiescent (healed) nor a relapse (reinflamed) state were studied. If there was a suggested loss of inhibitory neural control it is likely that these changes affect healing and the muscles' reaction to reinflammation. This would contribute to the abnormal motility pattern seen in clinically relevant conditions of IBD and chronic colitis.

RATIONALE

The present study investigated colonic smooth muscle responses to tachykinins in a novel model of inflammation, namely a 4 week healed and a reinflamed state. Specific studies include: 1) changes in the response of colonic circular smooth muscle to tachykinins during acute (4 hour) and chronic (4 week) inflammation and 2) NO's role in control, acute, healed, and reinflamed conditions.

HYPOTHESIS I

The healed (quiescent) and reinflamed state result in alterations in the response of circular smooth muscle to tachykinins in the rat distal colon that are different from those seen in control or acutely inflamed states.

HYPOTHESIS II

Nitrergic neurotransmission plays an important inhibitory role in colonic smooth muscle contraction under normal conditions that may be altered during inflammation and thereby result in changes to the neural control of smooth muscle responses to neurotransmitters.

MATERIALS AND METHODS

ANIMALS

Experiments and animal care were conducted in compliance with guidelines outlined by the "Guide for the Care and Use of Laboratory Animals" (Institute of Animal Research, National Research Council). Male, Sprague Dawley rats (Taconic Farms, 200-400 gms) were assigned at random to one of four treatment groups; control, acute, healed or reinflamed. After an overnight fast (water ad lib), rats were anesthetized with 8 mg/kg rompun (Miles Inc., Shawnee Mission, KS) in combination with 40 mg/kg ketamine (Fort Dodge Laboratories, Fort Dodge, IA), intramuscularly. The control group received 1 ml saline intra-rectally, while acute, healed and reinflamed animals were given 100 mg/kg trinitrobenzenesulfonic acid ; TNBS (Sigma, St. Louis, MO) in a 50% ethanol solution intra-rectally. Four hours later, control and acute animals were reanesthetized for surgical removal of a 4-5 cm section of distal colon (mid transverse to distal portion). These rats were euthanized with an overdose of pentobarbital(l0 mg/kg). Healed and reinflamed animals were allowed to recover and were monitored for 4 weeks. After four weeks, these rats were reinjected intra-rectally with either 1 ml saline (healed) or 100 mg/ml TNBS in 50% ethanol (reinflamed) and tissue harvested 4 hours later as observed previously for control and acute animals.

HISTOLOGY

Full thickness sections of distal colon from each animal were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with either hematoxylin and eosin (H & E), or Giemsa. In addition, mucosa free sections of distal colon from each group were fixed in 4% paraformaldehyde in phosphate-buffered solution (PBS) for visualizing NO synthase (NOS) NADPH diaphorase activity using the method of Shuttleworth, et al. (1993). A qualitative assessment was made as to presence or absence of NOS activity within enteric nerves and myenteric plexus. Sections were also stained with the lectin, Ulex Europaeus I (UEAI), which binds to fucose residues for the evaluation of mucus.

For muscle characteristics, tissue integrity and the thickness of muscularis externa, circular muscularis externa, muscularis mucosae, and submucosa were measured on H & E sections at 20X objective on light microscopy with an ocular micrometer (Olympus, Olympus Optical Co., LTD, Tokyo, Japan). At least three measurements were taken and values averaged. The number and distribution of inflammatory cells were evaluated in Giemsa stained sections. Changes in muscle thickness, submucosal thickening, and inflammatory cells were compared among the four treatment groups using a one way ANOVA followed by Bonferonni test where applicable.

in-vitro MEASUREMENT

Mucosa-free segments were mounted in organ baths in their circular axis. Segments were secured at each end with 4-0 silk and maintained in an oxygenated Krebs solution at

37˚C throughout the experiment. Krebs solution contained (in mM) 118.5 NaCl, 4.75 KCl, 2.54 $CaCl_2$, 1.19 $MgSO_4$, 25 $NaHCO_3$, 1.19 NaH_2PO_4 and 11.0 dextrose. Using methods described by Percy, (1996) the length of maximal active tension (L_0) was determined using Ach (10^{-4} M). Passive, active and total tension were also measured and recorded. Tensions were recorded on a Grass model 79 Polygraph (Grass Instruments, Quincy, MA). After L_0 determination tissues were allowed to equilibrate for at least 10 minutes.

In the appropriate experiments, receptor agonists and antagonists were added to the baths after a minimum of 10 minute equilibration post L_0. For each chamber that received an antagonist the blocker was added every 10 minutes after old buffer was flushed through the bath and fresh buffer added. Baths were incubated with the antagonist for 30 minutes prior to, and between, tachykinin addition. From each treatment group, 5-8 strips were used to determine Ach concentration response curves and another 5-8 strips used for KC1 concentration response curves. Ach was added noncumulatively, to muscle strips in log increments from 10^{-9} to 10^{-3} M. A stock solution of Ach (10^{-1} M) in distilled water was made and stored at 4 ˚C, and appropriate dilutions made fresh daily as needed. KC1 solution was made from a 4 M saturated stock solution and serial dilutions were made from 3 to 180 mM in distilled water.

Antagonists were made fresh daily. Antagonists used were; atropine sulfate (1 µM), hexamethonium (HEX, 10 µM), N^G nitro-L-arginine (L-NNA, 1 µM), aminoguanidine (AG, 1 µM), apamin (0.1 µM), and tetrodotoxin (TTX, 0.1 µM). Atropine, hexamethonium, aminoguanidine and apamin were dissolved in distilled water and chilled. L-NNA was dissolved in distilled water and maintained at 37°C. TTX was

prepared in a (50 mM citric acid, 48 mM NaH_2PO_4) and chilled.

The tachykinins were dissolved in 0.01 M acetic acid and stored as stock solutions of 10^{-4} M at -80˚C All chemicals except the tachykinins were obtained from Sigma (St. Louis, MO). Tachykinins were purchased from Peninsula (Belmont, CA). All muscle strips were challenged noncumulatively with SP or NKA in log increments from 10^{-9} to $l0^{-6}$ M. TNBS was obtained from Sigma as a 5% aqueous solution of picric acid.

Muscle strips from each of the four treatment groups received the tachykinins SP and NKA first without antagonists and then in separate experiments using one of the following combinations of receptor agonists, and/or antagonists;

1. atropine (muscarinic Ach blocker)
2. L-NNA (nonspecific NOS inhibitor)
3. L-NNA + atropine
4. L-NNA + apamin (K channel blocker)
5. aminoguanidine (specific iNOS inhibitor)
6. hexamethonium (ganglionic blocker)
7. tetrodotoxin (Na channel blocker used to block nerve conduction)

In addition, a complete list of neurotransmitters, their antagonists, and mechanisms of action for each also appears in the appendix.

At the end of each experiment all muscle strips were challenged first with Ach 10^{-3} and then 60 mM KC1 to confirm neuronal and smooth muscle viability respectively. Finally, muscle strips were weighed. Muscle tension was normalized to cross sectional

area according to methods described by Percy, (1996), using the formula:

$$\text{Force/area} = \frac{\text{grams tension developed X 9.8 m/sec}^2}{\{[\text{mass (gm wet weight) x 0.726}]/[1.05 \text{ x } (L_o)]\}}$$

where mass was corrected for water content by multiplying the wet weight obtained by 0.726, and 1.05 is the muscle density .

Other measurements calculated throughout the experiments included EC_{50}'s or concentration at which 50% of the maximal tension was developed in response to an agonist and antagonist. Concentration response curves were generated using a best fit regression curve for mean responses at each concentration for an experimental group. The EC_{50}'s and concentration response curves for the agonists were plotted using Prism Graphpad software (Graphpad, Inc., Philadelphia, PA). In addition, spontaneous contractions were measured on muscle strips from the four treatment groups after L_0 prior to, and after L-NNA and AG antagonist. Specifically, amplitude as tension (mN/cm^2), frequency as number of contractions per 10 min, and duration of contraction (seconds) were recorded and comparisons between groups noted.

STATISTICS

Results were summarized as mean $\pm$ standard error of the mean. Analysis of variance was performed on all concentration response curves from the four treatment groups. Significant differences for singular measurements between control and inflamed groups were performed by post hoc comparisons tests such as Bonferoni t-test using Prism Graphpad software (Graphpad, Inc., Philadelphia, PA). Multiple ANOVA statistical tests were determined with statistical package program Systat 5.2 (Systat Inc.).

RESULTS

ANIMALS

TNBS inflammation induced a transient diarrhea that resolved within one week. Over 2-3 weeks animals showed a change in stool characteristics from a soft semiformed stool to more normal characteristics by 4 weeks. All healed and reinflamed animals had normal appetite, gained weight and except for the initial diarrhea, appeared normal.

MORPHOLOGY and MUSCLE PARAMETERS

HISTOLOGY

Morphological measurements and comparisons for the four treatment groups are shown in Figures 2a-c. Changes seen in acute colonic tissue included pronounced vasocongestion, submucosal thickening, and a predominantly neutrophilic infiltration as expected. In addition, mild sloughing of the mucosal layer was noted in acute tissue that was even more profound in the reinflamed tissue. Healed tissue measurements resembled control tissue in both circular and muscularis muscle layers, and submucosal thickness. Reinflamed tissue had a significant increase of submucosal thickening when compared to the healed tissue. In addition, reinflamed tissue showed moderate to severe damage to crypt regions that was not evident in acute inflammation.

Acutely inflamed tissue had more numerous infiltrates of PMN's when compared to control tissue. Results of polymorphonucleocyte counts in the four treatment groups are illustrated in Figure 2d. Both healed and reinflamed tissue had greater lymphocytic type infliltrates. H & E photomicrographs of sections from the four treatment groups are

FIGURE 2

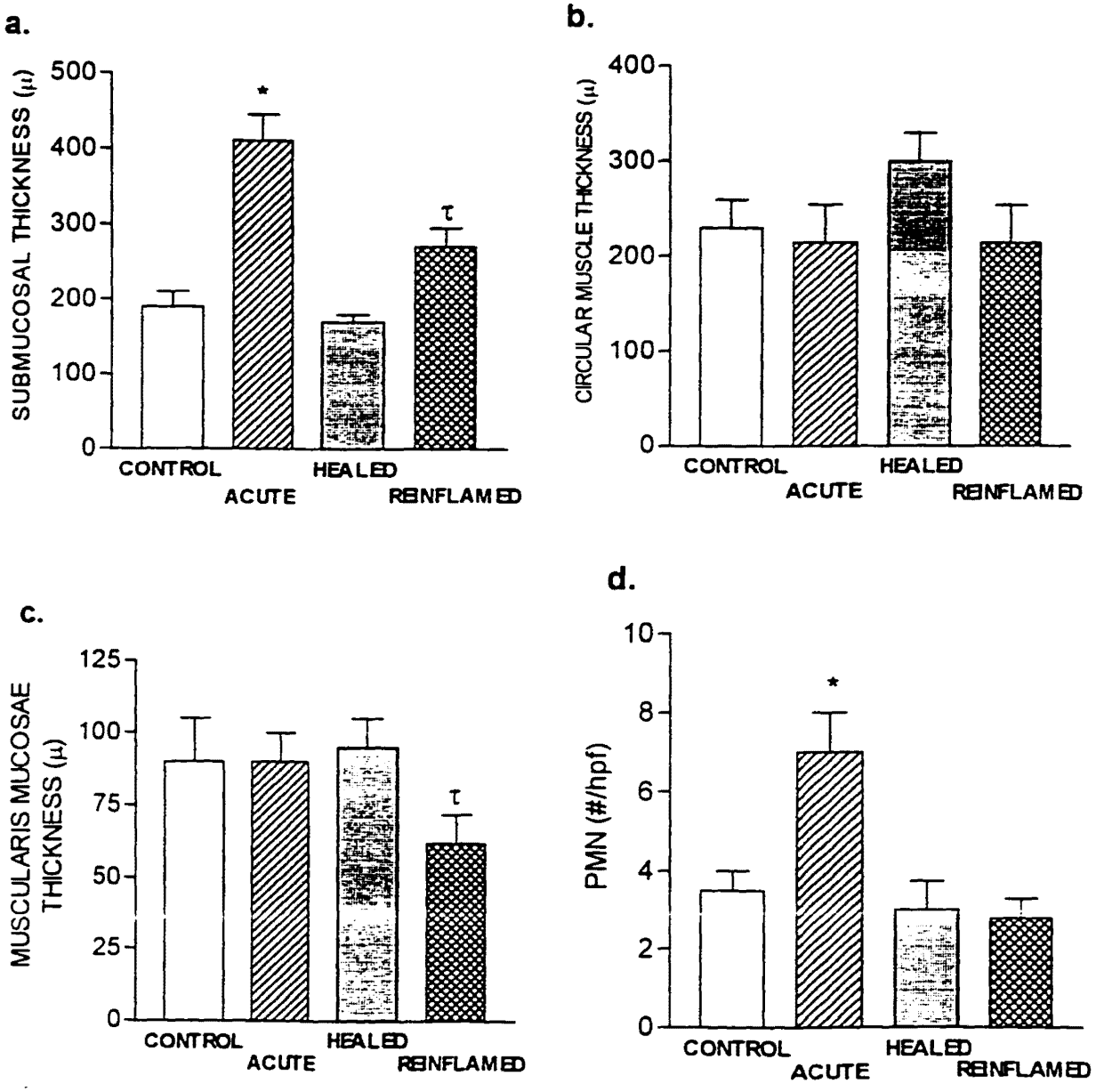


Figure 2. Morphological measurements (a-c) and PMN counts (d) for the four treatment groups. * $p < 0.05$ from CONTROL, $^{\tau}$ $p < 0.05$ from ACUTE

shown in Figure 3. Healed and reinflamed tissue showed a greater accumulation of mucus within the villi and/or lumen when compared to tissue from the control and acute animals. Photomicrographs of sections from the four treatment groups stained with the lectin are shown in Figure 4. Mucus production increased during all stages of inflammation. Assessment of NOS activity showed loss of NOS staining characteristics in acute, healed and reinflamed tissue. Examples of NOS stained photomicrographs are shown in Figure 5.

MUSCLE PARAMETERS

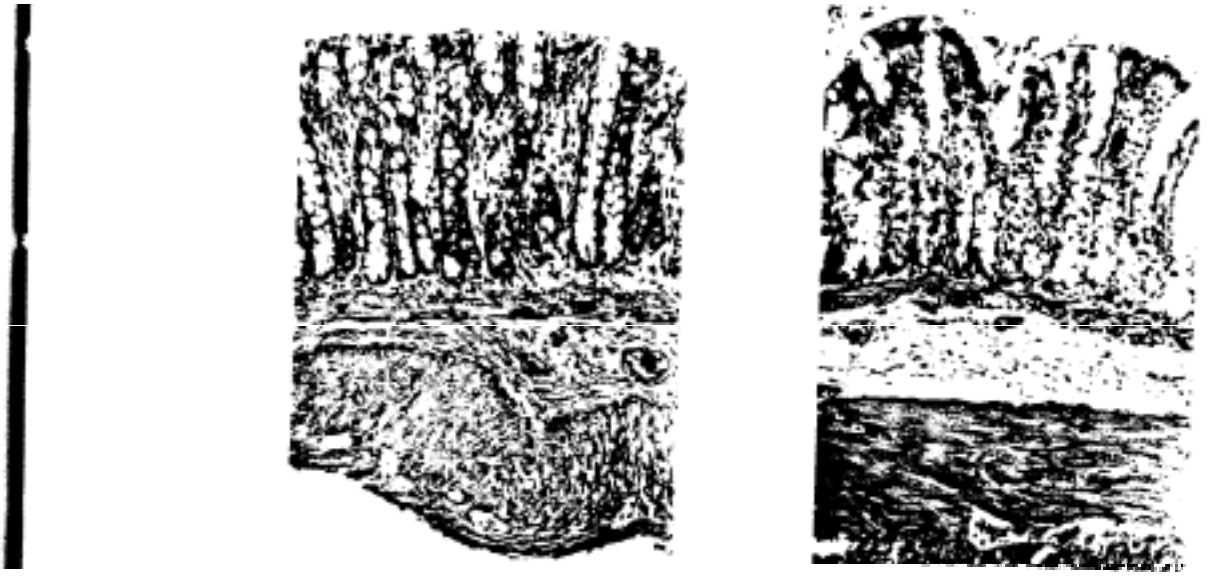
Physical characteristics of the muscles from the four treatment groups used in isometric contractions are summarized in Table la. No differences in surface area measurements were noted between control and acutely inflamed muscle however, healed and reinflamed muscles showed significant increases in both L_0 and wet weight when compared to control. Tensions at L_0 and in response to Ach (10^{-4}) are shown in Table 1b. No differences in passive, active, or total tensions were found in any of the four treatment groups.

MUSCLE CONTRACTION

SPONTANEOUS CONTRACTIONS

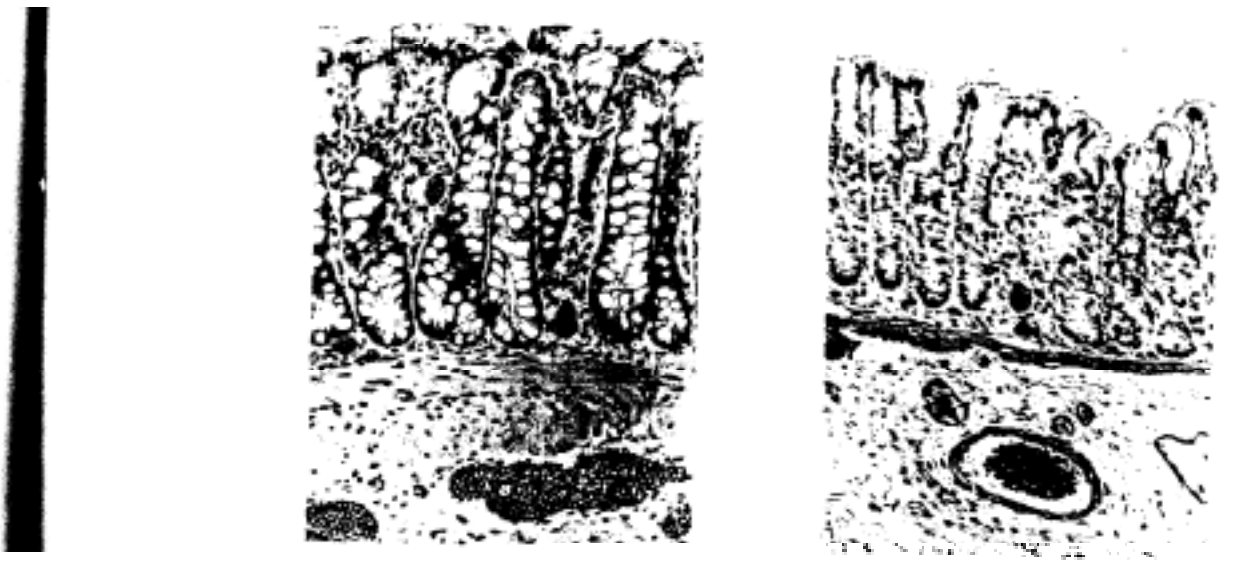
Inflammation (acute and reinflammation) induced a significant increase in the amplitude of spontaneous contractions (Figure 6a and Table 2). In control and healed tissues, addition of the nonspecific NOS inhibitor, L-NNA further increased the amplitude of spontaneous contractions from vehicle treated tissue. Acute and reinflamed tissue demonstrated no further increase in amplitude from their baseline values with

FIGURE 3. Photomicrographs of H & E stained tissue of distal colon from control, acute. healed and reinflamed male Sprague Dawley rats. (Magnification for control. acute and reinflamed at 1OX. healed at 4OX).



CONTROL

ACUTE



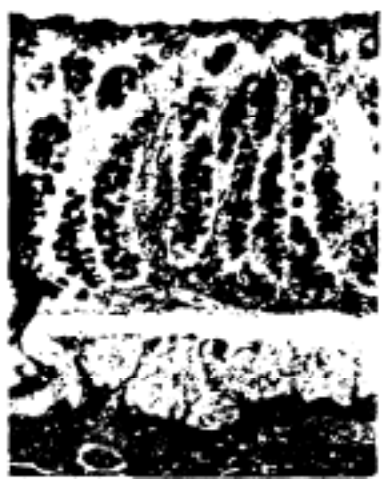
HEALED

REINFLAMED

FIGURE 4. Photomicrographs of Lectin stained tissue sections of distal colon from control, acute, healed and reinflamed male Sprague Dawley rats. (Magnification at 10X).

CONTROL

ACUTE



HEALED

REINFLAMED

FIGURE 5. Photomicrographs of NOS stained tissue sections of distal colon from control, acute, healed and reinflamed male Sprague Dawley rats. (Magnification at 10x).

CONTROL

ACUTE

HEALED

REINFLAMED

Table l a

Surface Area Measurements

	L_o (cm)	Wet weight (mg)
Control	1.12 ± 0.076	2.3 ± 0.05
Acute	1.20 ± 0.042	2.6 ± 0.37
Healed	1.46 ± 0.030*	6.0 ± 0.38*
Reinflamed	1.52 ± 0.047*	6.1 ± 0.23*

mean ± s.e.m., * $p < 0.05$ from Control

Table l b

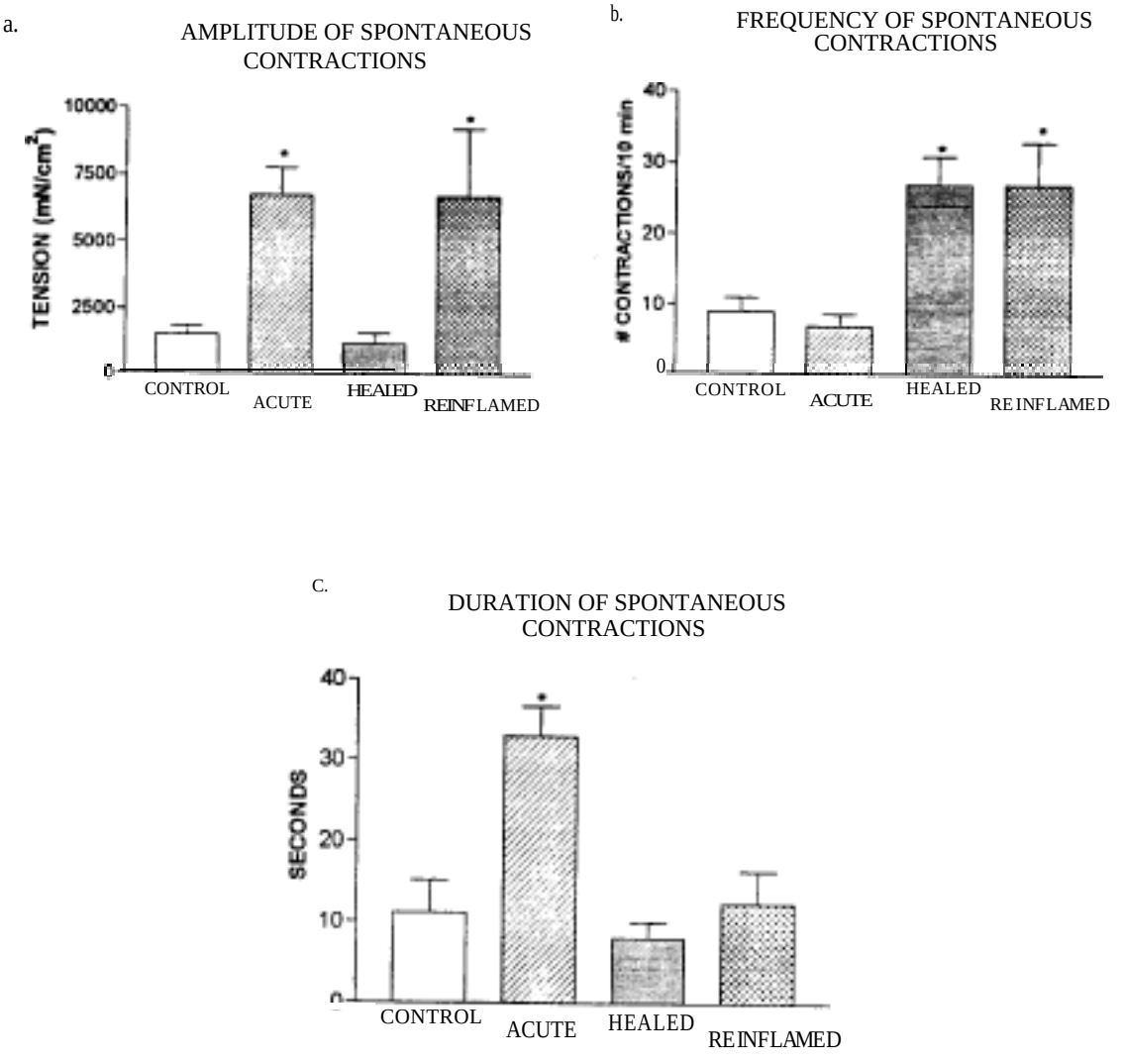
Tension Measurements (mN/cm^2)

	Passive[1]	Active[2]	Total[3]
Control	11437 ± 1634	25266 ± 1926	36703 ± 1772
Acute	8527 ± 1149	24970 ± 2444	33498 ± 2937
Healed	10495 ± 1161	19375 ± 2306	29870 ± 2758
Reinflamed	14912 ± 3066	21779 ± 2076	36691 ± 4455

mean ± s.e.m.

1. Tension developed in muscle after L_o determination.
2. Tension generated in the muscle after stimulation by Ach (10^{-4})
3. Total of passive and active tension after stimulation by Ach (10^{-4})

FIGURE 6



* $p < 0.05$ from CONTROL

FIGURE 6. Spontaneous Contractions during inflammation in vehicle treated smooth muscle. Results are mean ± SEM.

Table 2. Spontaneous contractions for the four treatment groups in the absence (vehicle) and presence of NOS inhibitors, L-NNA and AG.

TREATMENT	VEHICLE	+ LNNA	+AG
CONTROL			
amnlitude (tension)	1529 ± 336.8	5294 ± 569.5	3529 ± 896.7
frequency (cycles/l0 min)	8.8 ± 2.45	29 ± 3.89	4.7 ± 0.8 1
duration (seconds)	11. 2± 3. 59	12.9± 3.08	9.6 ± 1.57
ACUTE			
amnlitude (tension)	6755 ± 1003.8*	5616.2 ± 1274.6	7247 ± 1466.2
frequency (cycles/l 0 min)	7.1 ± 1.62	8.4 ± 0.80*	11.7 ± 2.39*
duration (seconds)	32.9 ± 3.69*	32.3 ± 3.47*	28.3 ± 3.53*
HEALED			
amplitude (tension)	1225 ± 375.2	5000 ± 1183.0§	2696 ± 970.7
frequency (cvcles/l0 min)	27.0 ± 4.35*	21.3 ± 5.09	4.7 ± 1.96
duration (seconds)	7.89 ± 2.38	15.6 ± 1.76	15.4 ± 3.17
REINFLAMED			
amplitude (tension)	6705 ± 2526.1	6614 ± 1516.4	3516 ± 1175.0
frequency (cycles/ l0 min)	26.8 ± 6.15†	11.3 ± 1.96	4.25 ± 1.21
duration (seconds)	12.4 ± 4.12†	22.7 ± 1.88†	13.2 ± 3.45

§, $p < 0.05$ from VEHICLE: *, $p < 0.05$ from CONTROL treatment: †, $p < 0.05$ from ACUTE treatment.

addition of L-NNA. These effects suggest that NO exerts a tonic inhibition of amplitude that is lost during TNBS-induced inflammation. Table 2 and Figure 7a show the result of changes seen in amplitude in the presence of L-NNA.

The frequency of spontaneous contractions was unchanged during the initial inflammation (acute), but was elevated in the healed and reinflamed groups. L-NNA increased frequency in control but not in acute and healed tissue. There was a decrease in frequency of contractions with L-NNA in reinflamed tissue. Table 2, Figure 6b, and Figure 7b illustrate these changes in frequency. Aminoguanidine caused no change in frequency of spontaneous contractions in control and acute tissue and a decrease in frequency in healed and reinflamed tissue. Table 2, Figure 6 and Figure 7c show these changes in the presence of AG.

The duration of colonic smooth muscle contractions was increased only by acute inflammation and was not affected by L-NNA or AG. These results are shown in Table 2, and Figure 6c.

These results suggest that constitutive NO controls the amplitude and frequency of spontaneous contractions in control tissue. During inflammation (acute and reinflammation) there is a temporary increase in the amplitude of spontaneous contractions and after acute inflammation there is a lasting increase in frequency of spontaneous contractions. This is consistent with the loss of NOS staining and would imply a loss of cNOS control and/or a gain in iNOS regulation of the frequency of spontaneous contractions.

FIGURE 7

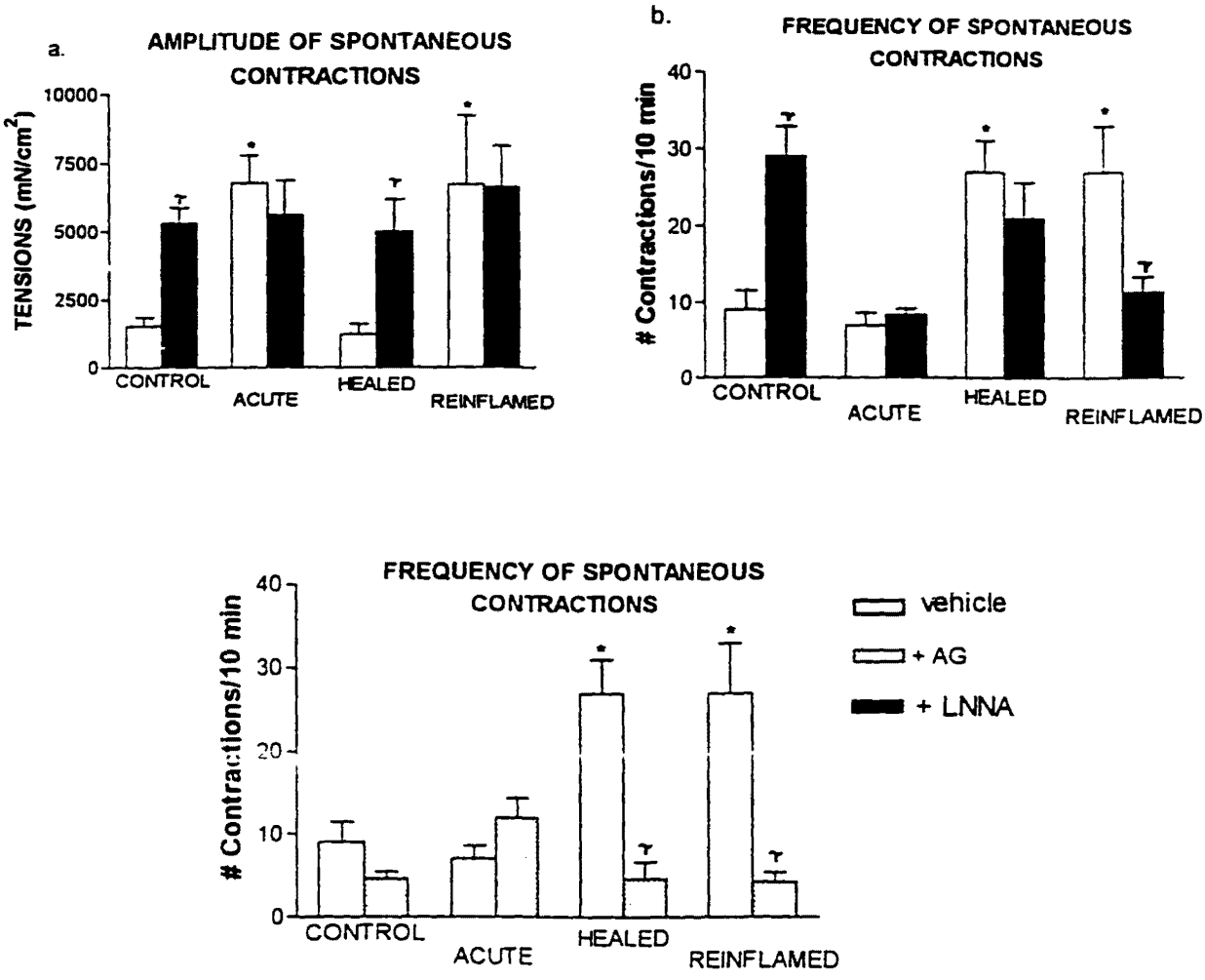


FIGURE 7. Spontaneous contractions during inflammation in the presence and absence of L-NNA (a and b) or AG (c). Results are mean ± SEM.

CONCENTRATION RESPONSE CURVES

KCL AND ACH

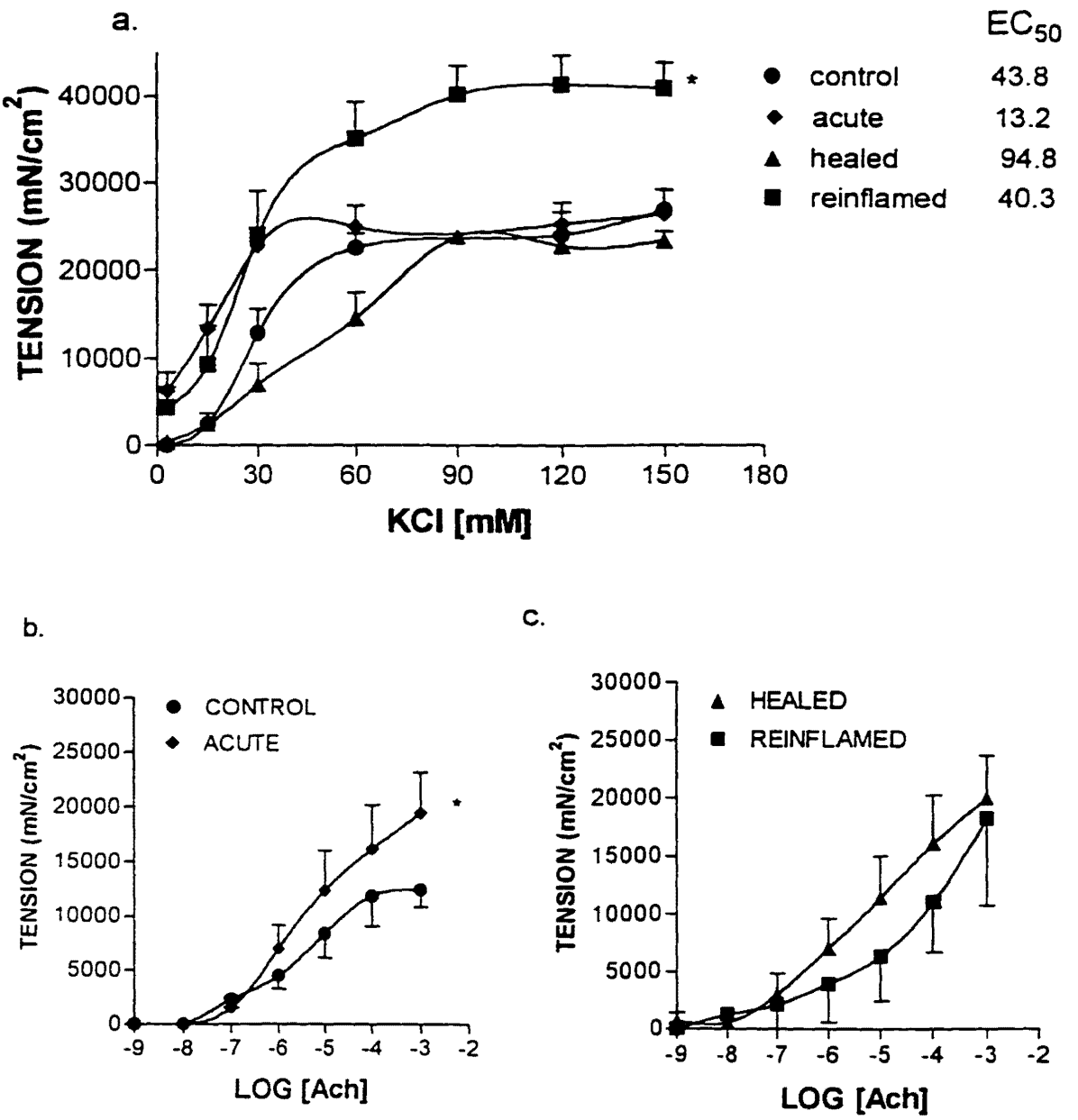
Concentration response curves to the depolarizing agent KC1 demonstrated that the acute inflammation increased sensitivity of smooth muscle to K depolarization, as shown by the lower EC_{50} value for this group (Figure 8a). The maximum response however, was not significantly different from control. Smooth muscle from healed colon also had a similar peak response to controls, but a reduced sensitivity, while reinflamed tissue gave similar sensitivity but a greater maximum response curve ($p < 0.05$).

Concentration response curves to Ach are shown in Figure 8b and c. Acute tissue demonstrated a significant increase in the response curve when compared to control ($p < 0.05$). Healed tissue showed no difference from control suggesting a return to pre-inflamed conditions, that remained when the tissue was reinflamed. There were no changes in sensitivity of the responses to Ach among the four treatment groups (EC_{50}).

SMOOTH MUSCLE RESPONSE TO TACHYKININS

TNBS-induced inflammation also changed the circular smooth muscle responses to substance P (SP) and neurokinin A (NKA). Figure 9 a-d compares the concentration response curves for SP and NKA for all treatment groups. Acute inflammation induced a significant increase in the response to SP when compared to control muscle ($p < 0.05$). In contrast, responses for NKA were significantly decreased for acute tissue when compared to control ($p < 0.05$). For both SP and NKA, healed and reinflamed muscles responses were unchanged from control.

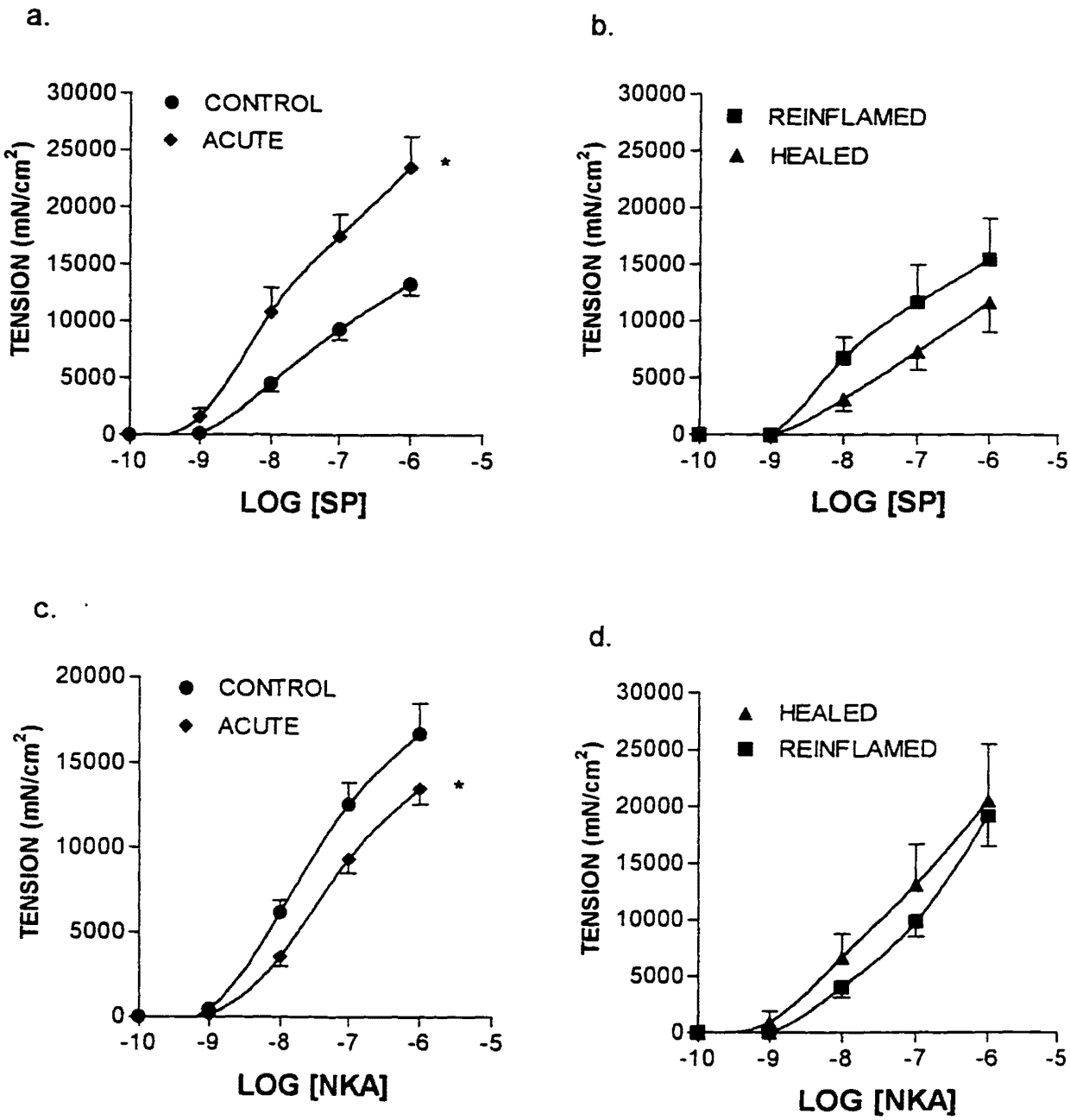
FIGURE 8



* $p < 0.05$ from CONTROL

Figure 8. KCl and Ach concentration response curves in untreated baths for the four treatment groups. Results are mean ± SEM.

FIGURE 9



* $p < 0.05$ from CONTROL

Figure 9. SP (a, b) and NKA (c, d) concentration response curves in untreated baths for the four treatment groups. Results are mean ± SEM.

Experiments thus far focused primarily on changes seen during the stages of inflammation at the level of the muscle and nerves. Circular smooth muscle responses to SP and Ach were increased significantly, while responses to NKA were decreased significantly during acute inflammation. The response of healed tissue to Ach and to both tachykinins were unchanged from controls. In contrast, reinflamed tissue remained unchanged in its response to Ach, SP, or NKA, when compared to healed. The responses to these excitatory neurotransmitters in reinflamed did not mimic the response pattern seen in acute inflammation. Following these experiments, the influence of different neurotransmitters on tachykinin responses under control conditions and throughout inflammation was then assessed with the use of antagonist agents.

CONTROL

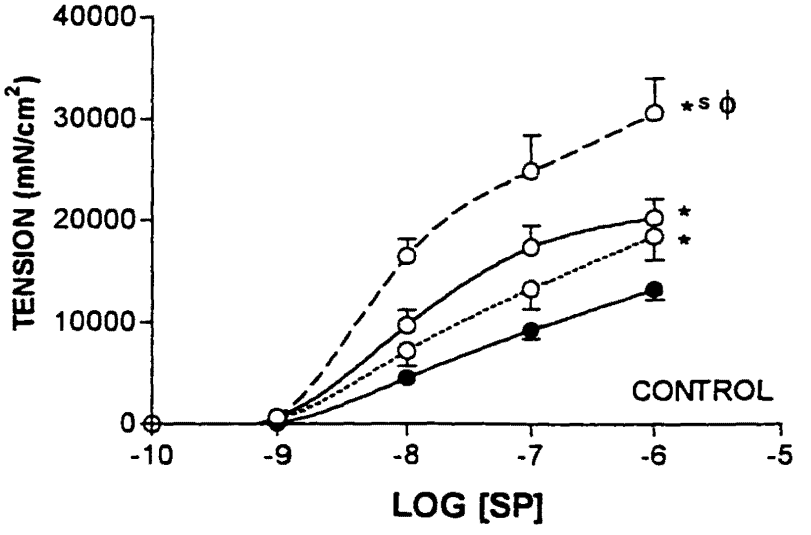
Studies utilizing atropine to evaluate the response of muscarinic mediated Ach release were conducted previously within this laboratory (Hosseini, 1996). These experiments combined with those reported in this study revealed a significant increase in the response to SP in the presence of atropine, suggesting that SP's action was normally inhibited by input from muscarinic receptors. In contrast, there was no change in the response to NKA with the addition of atropine. Table 3 and Figure 10a and b summarize these results.

To further elucidate the effect of an inhibitory neurotransmitter on control muscle, the nonspecific NOS inhibitor, L-NNA, was used to examine the consequent action of the loss of NO on smooth muscle contractility. Table 3 and Figure 10a and b summarize the effects of L-NNA on the response in control muscle to the tachykinins. Like atropine, L-NNA significantly increased the response to SP but had no effect on the response to NKA.

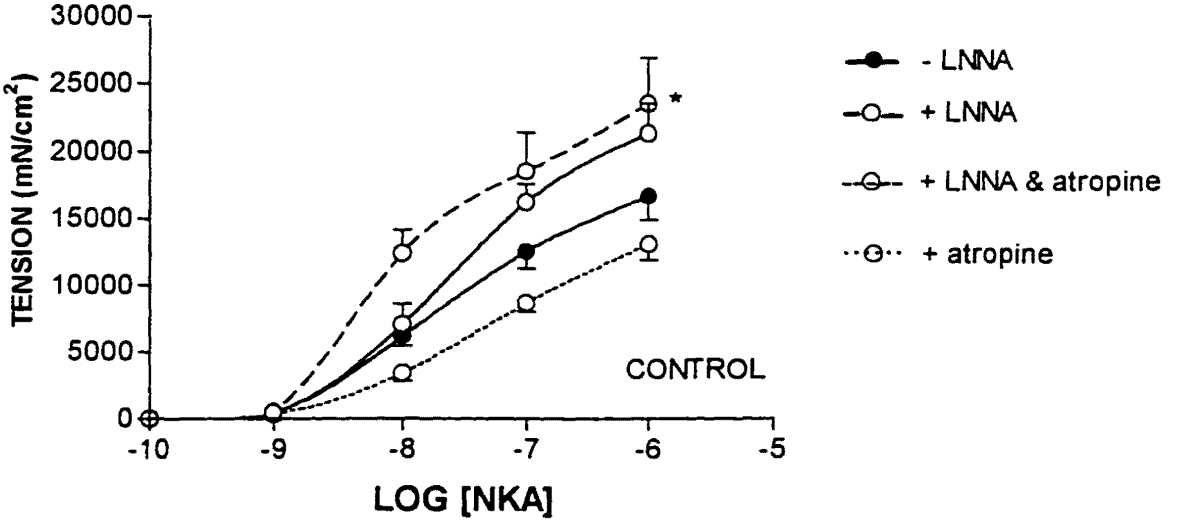
To determine whether NO's action was within the same or different pathway's used by Ach, the smooth muscle was incubated with both L-NNA and atropine and the response compared the response to those seen in the presence of a single agent. Results shown in Figure 10a and b and Table 3 showed a significant increase in both tachykinins response in the presence of L-NNA and atropine . In addition, there was also a significant difference between responses to SP for muscle incubated with only L-NNA or atropine compared to that of the L-NNA and atropine combination. This supports the hypothesis that there is both a chohnergic and nitrergic control on the smooth muscle and that they most likely work via separate pathways to inhibit SP's action on smooth muscle. In

FIGURE 10

a.



b.



* $p < 0.05$ from - LNNA

^{s} $p < 0.05$ from + atropine

$^{\phi}$ $p < 0.05$ from + LNNA

FIGURE 10. SP (a) and NKA (b) concentration response curves for CONTROL in the presence and absence of L-NNA, atropine, or L-NNA + atropine. Results are mean ± SEM.

Table 3: Summary of peak responses of tachykinins ± antagonists in inflamed rats.

	CONTROL		ACUTE		HEALED		REINFLAMED	
	SP	NKA	SP	NKA	SP	NKA	SP	NKA
vehicle	13270 ± 983	16665 ± 1759	23472 ±2751'	13444 ± 915'	11726 ± 2613	20478 ± 4990	15537 ± 3600	19149 ± 2691
+ atropine	* 18531 ± 2394	13087 ± 1203	22206 ± 4961	* 21413 ± 3246	* 21309 ± 4055	20929 ± 4644	* 24569 ± 4486	18904 ± 3445
+ Lnna	* 20320 ± 1850	21333 ± 2200	24133 ± 2199	* 21342 ± 2368	* 24771 ± 2468	25739 ± 2815	* 29037 ± 2997	* 25149 ± 4153
+lnna+ apamin	*ϕ 37868 ± 3111	*ϕ 28035 ± 4091	+ 24214 ± 1067	* 28877 ± 3854	*ϕ 31029 ± 2832	*ϕ 31654 ± 3095	*ϕ 38840 ± 3470	*ϕ 36010 ± 3548
+ AG	* 23407 ± 2134	* 23542 ± 1451	*ϕ 31945 ± 2109	* 26751 ± 2565	* 23935 ± 2659	23235 ± 2549	* 26147 ± 1780	* 32977 ± 3726
+ HEX	* 27902 ± 3162		28486 ± 3765		* 23373 ± 2426		* 31725 ± 2480	
+ lnna+ atropine	*ϕ§ 30668 ± 3415	* 23541± 3397	* 34695 ± 2540	*ϕ§+ 35887 ± 3119	* 24658± 3365	*ϕ§+ 33905 ± 3241	* 32169 ± 2785	*§ 30275 ± 1266
+ TTX	* 30657 ± 3778	* 24712 ± 3349	* 34503 ± 3312	* 30878 ± 3310	* 33344± 2697	26593 ± 3022	* 35823 ±2756	* 33249± 4517

mean peak value ± sem, * $p \leq 0.05$ from vehicle, + $p \leq 0.05$ from control, ϕ $p \leq 0.05$ from + lnna, § $p \leq 0.05$ from + atropine
n = 5-15 strips

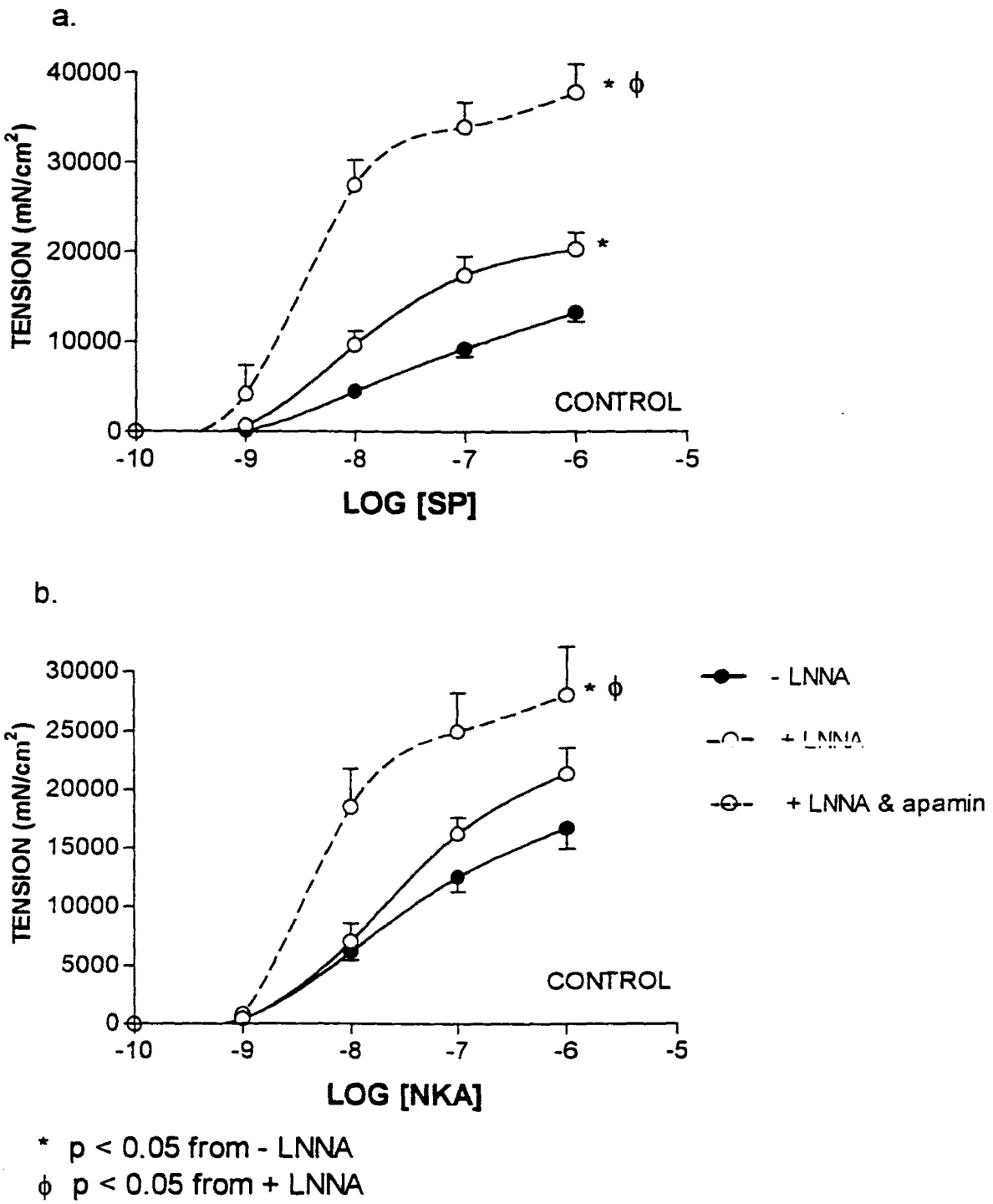
contrast, NKA responses demonstrated only the additive effect of cholinergic and nitrergic control, since an increase was observed only when the two antagonists were added together.

Finally the role of inducible NO was investigated with the use of aminoguanidine (AG), a selective iNOS inhibitor. AG increased responses to both SP and NKA in all treatment groups. Peak responses for this drug are shown in Table 3.

Following these experiments on the effects of NO on smooth muscle contractility, other possible inhibitory neurotransmitters were evaluated. This included the effects of NO as well as neurotransmitters that act on K^+ channels resulting in hyperpolarization of the cell and consequent relaxation. Using apamin, a K^+ channel blocker, in combination with L-NNA, the influence of any inhibitory signal to the smooth muscle can be blocked effectively. Figure 11a and b, and Table 3 summarize these results. Both tachykinins' response curves were significantly increased compared to control values. In addition there was a significant difference from that of muscles incubated with L-NNA alone, suggesting significant contribution of other inhibitory neurotransmitters utilizing K channels to the overall contractile state of the muscle.

To demonstrate the use of neural and ganglionic pathways for the transmission of these inhibitory neurotransmitters, tetrodotoxin and hexamethonium were used. The results of the Na^{+2} channel blocker tetrodotoxin (TTX) and its effects on smooth muscle contractility to the tachykinins are shown in Figure 12a and b and Table 3. These results show a significant increase from basal/vehicle conditions for both tachykinins, suggesting that SP lead to release of inhibitory neurotransmitters. Results for HEX, the ganglionic blocker, were also increased from basal/vehicle conditions. These results shown in Table

FIGURE 11



* $p < 0.05$ from - LNNA

ϕ $p < 0.05$ from + LNNA

FIGURE 11. SP (a) and NKA (b) concentration response curves for CONTROL in the presence and absence of L-NNA or L-NNA + apamin.
Results are mean ± SEM.

FIGURE 12

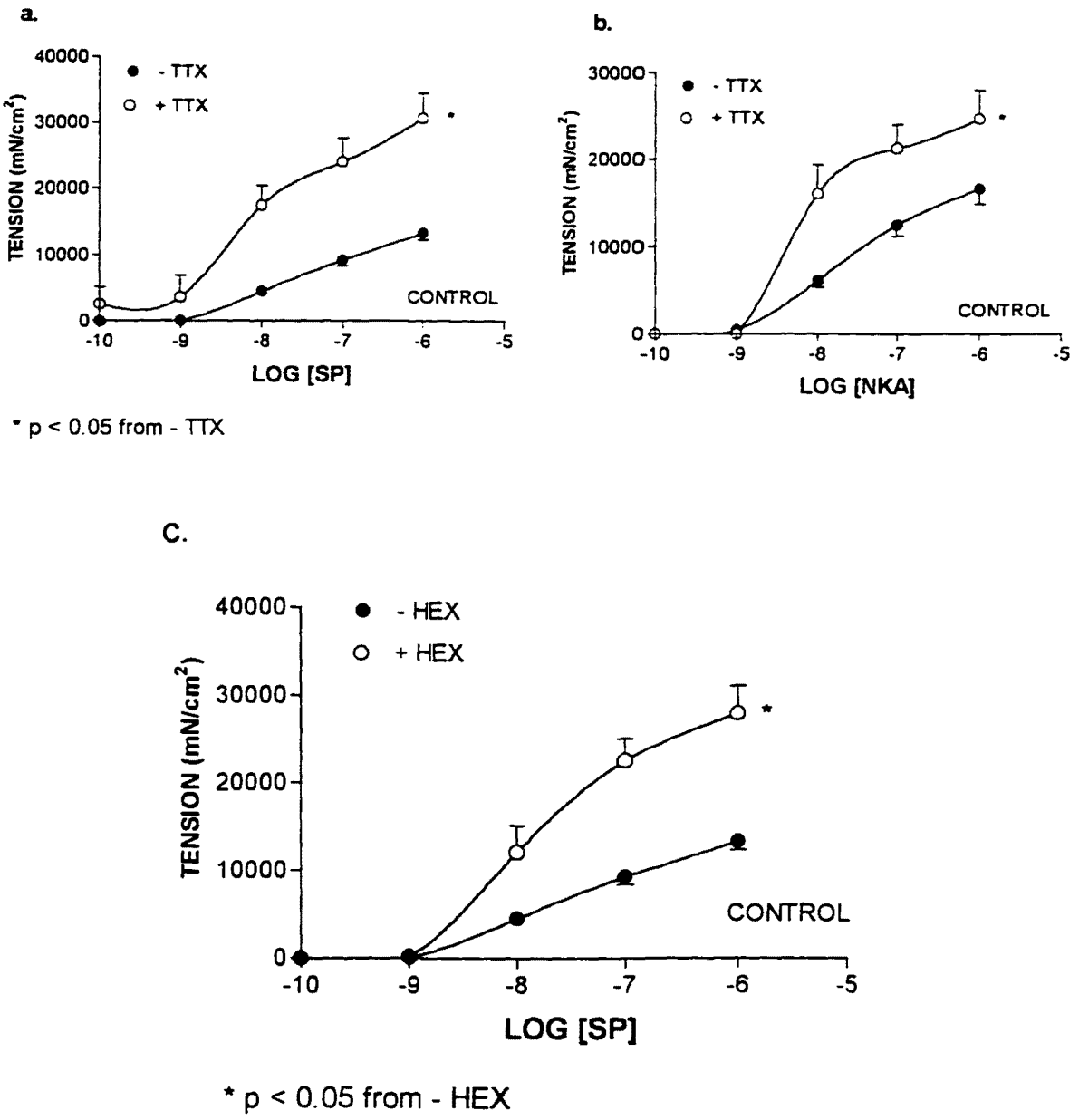


FIGURE 12. SP (a, c) and NKA (b) concentration response curves for CONTROL in the presence of TTX (SP and NKA) and HEX (SP). Results are mean ± SEM.

3, and Figure 12c for SP, suggest that ganglionic innervation also has significant effects on relaxation of the smooth muscle.

In summary, under control conditions the innervation to normal smooth muscle of the colon exhibits both chohnergic and nitrergic control that act via separate pathways. There is also a significant influence of other inhibitory neurotransmitters utilizing K^+ channel hyperpolarization. All these pathways appear to be tetrodotoxin- and hexamethonium-sensitive suggesting that SP work via nerves and/or ganglia, in addition to a direct action on smooth muscle leading to the release of an inhibitory neurotransmitter.

ACUTE

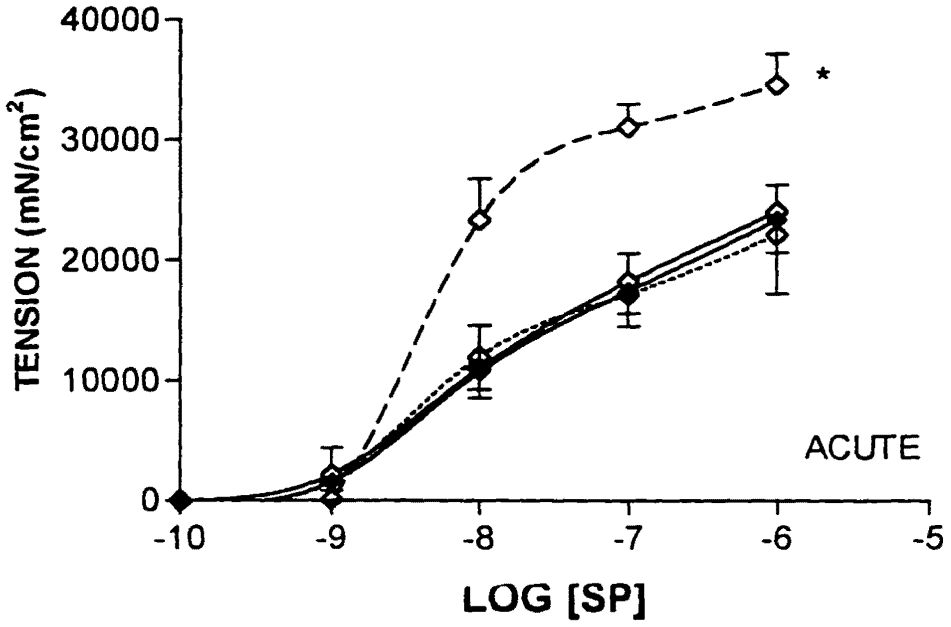
Acute inflammation caused a significant increase in concentration response to SP (Figure 9a) but a decrease in the response to NKA (Figure 9c). Atropine did not alter the response to SP (Table 3, Figure 13a) suggesting a loss of cholinergic inhibition in acute inflammation. In contrast, NKA responses, were significantly increased in the presence of atropine (Table 3, Figure 13b).

In the presence of L-NNA, smooth muscle from acutely inflamed colon caused no change in the response of smooth muscle to SP (Figure 13a and b and Table 3). Similar to the loss of muscarinic cholinergic inhibition shown previously, there was a loss of nitrergic control in acutely inflamed tissue. A significant increase in the response to NKA was found in the presence of L-NNA ($p < 0.05$), an effect that was not observed in the presence of LNNA in controls.

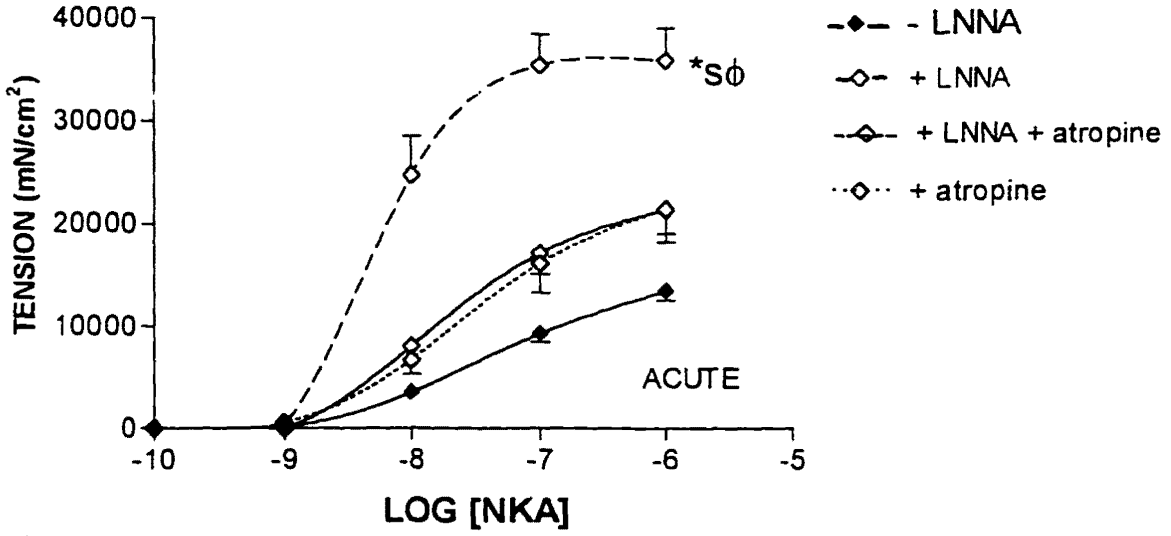
In the presence of L-NNA and atropine, acute tissue, like control, showed a

FIGURE 13

a.



b.



* $p < 0.05$ from - LNNA

^{s} $p < 0.05$ from + atropine

$^{\phi}$ $p < 0.05$ from + LNNA

FIGURE 13. SP (a) and NKA (b) concentration response curves for ACUTE in the presence and absence of L-NNA, atropine or L-NNA + atropine. Results are mean ± SEM.

significant increase in the response to both tachykinins ($p < 0.05$, Figure 13a, and b, Table 3). As was shown by the individual experiments outlined above, there was a loss of independent cholinergic and nitrergic control. In contrast, there was a significant increase seen when both antagonists were present indicating the additive effect of these two pathways was maintained as was noted in control.

To investigate the contribution of inducible NOS to the control of smooth muscle, concentration response curves to tachykinins were performed in the presence of aminoguanidine. The addition of aminoguanidine to smooth muscle from acutely inflamed colon caused a significant increase in the response to both tachykinins. In addition, there was a significant difference, between the response of SP in the presence of L-NNA versus AG. Table 3 summarizes the results of responses for the tachykinins in the presence of aminoguanidine.

Results for the responses of the tachykinins in the presence of L-NNA and apamin for acute tissue are shown Table 3 and Figure 14 a, and b. The ability of SP to release other inhibitory neurotransmitters remained unchanged in acute inflammation. Thus, in the presence of L-NNA and apamin as in saline controls, responses to SP remained elevated above those observed under basal conditions or with L-NNA alone. L-NNA and apamin resulted in an increase in the response of NKA, but this response was no different from that after the addition of L-NNA alone.

Similar to control, acute inflammation showed an increase in the response of both tachykinins to the addition of tetrodotoxin. In the presence of hexamethonium, the response to SP also remained elevated. Results for these two antagonists are shown in Table 3 and Figure 15 a, b, and c.

FIGURE 14

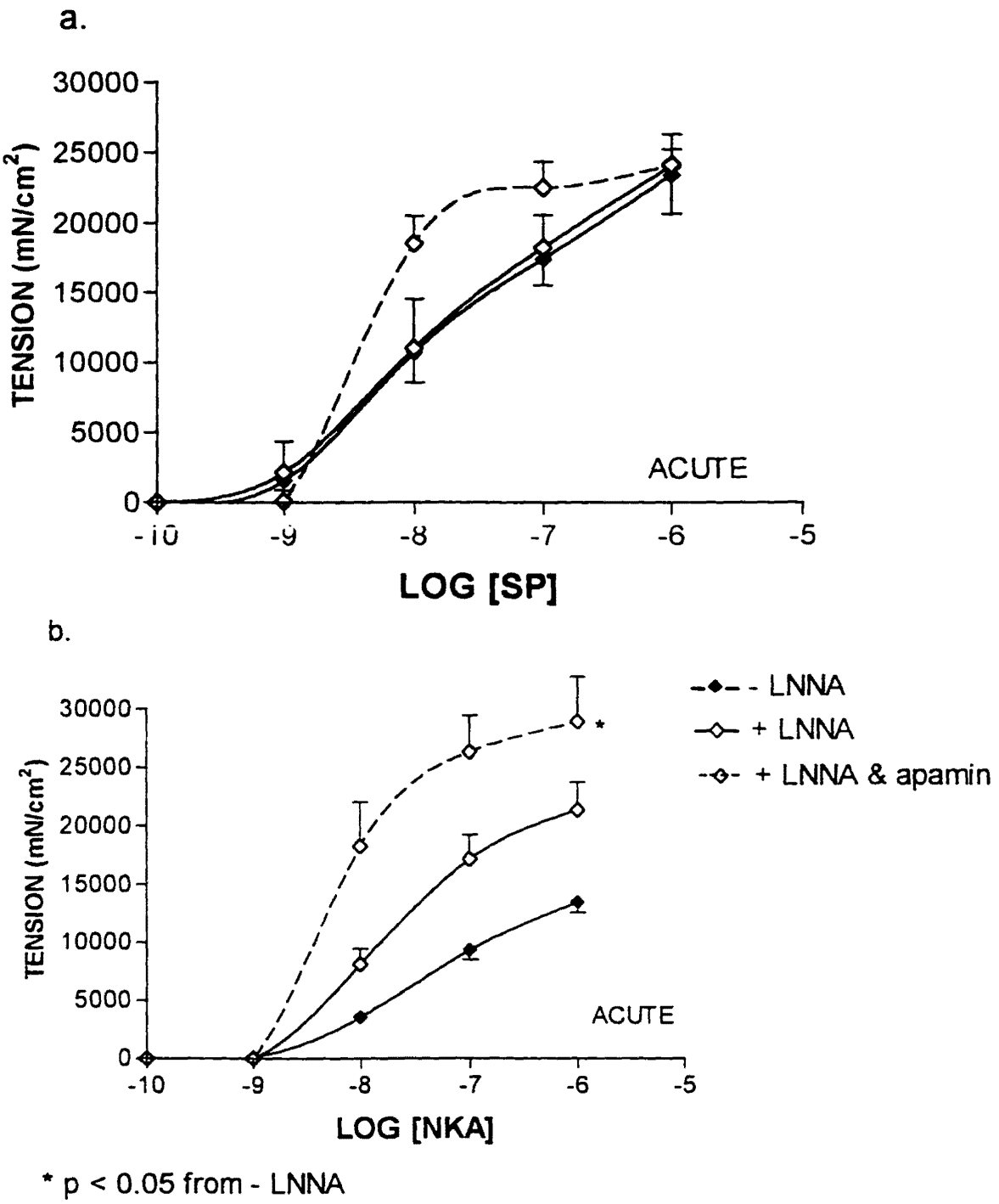
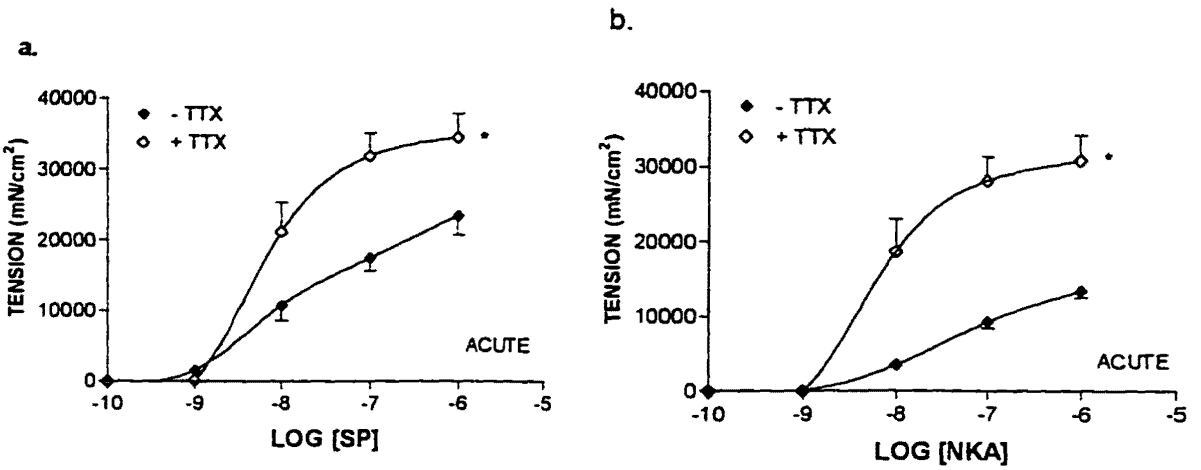


FIGURE 14. SP (a) and NKA (b) concentration response curves for ACUTE in the presence and absence of L-NNA or L-NNA + apamin. Results are mean + SEM.

FIGURE 15



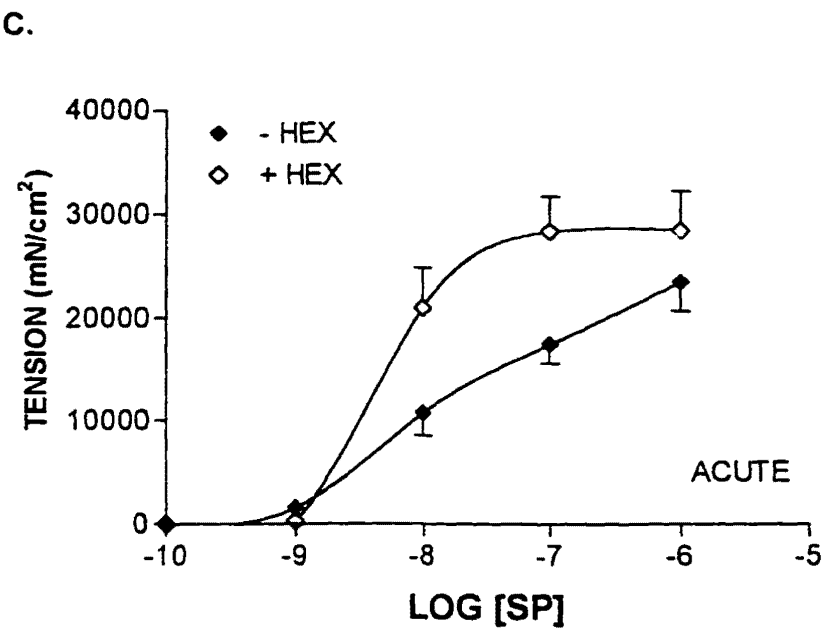


FIGURE 15. SP (a, c) and NKA (b) concentration response curves for ACUTE in the presence of TTX (SP and NKA) and HEX (SP). Results are mean ± SEM.

In summary, acute inflammation resulted in a loss of cholinergic and nitrergic inhibitory control. This result was consistent with the loss of NOS staining in this muscle. Acute tissue, however showed significant inhibitory control through the action of Ach and NO acting together, and through NO and other inhibitory neurotransmitters. The results with AG suggest that iNOS may contribute to the production of NO and to the overall inhibitory control.

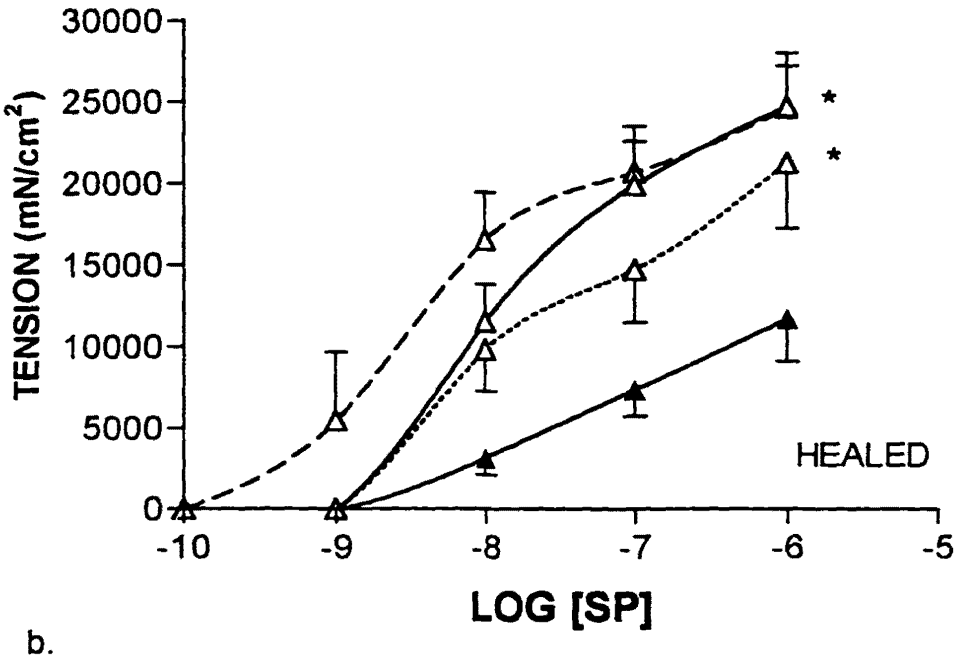
HEALED

Healed tissue was similar to control with respect to concentration responses of the tachykinins in the presence of various antagonists. The results of response curves for the tachykinins in healed muscles are summarized in Table 3 and Figure 9b, and d and were no different from control tissue. In the presence of atropine, healed tissue revealed similar responses as in controls, namely an increase in the response to SP and no change in the NKA response. These results are shown in Table 3 and Figure 16a and b. As in control, this suggested that SP is normally inhibited by Ach acting at muscarinic receptors. In addition, the similarity between control and healed responses suggests a return of inhibitory control by this mechanism.

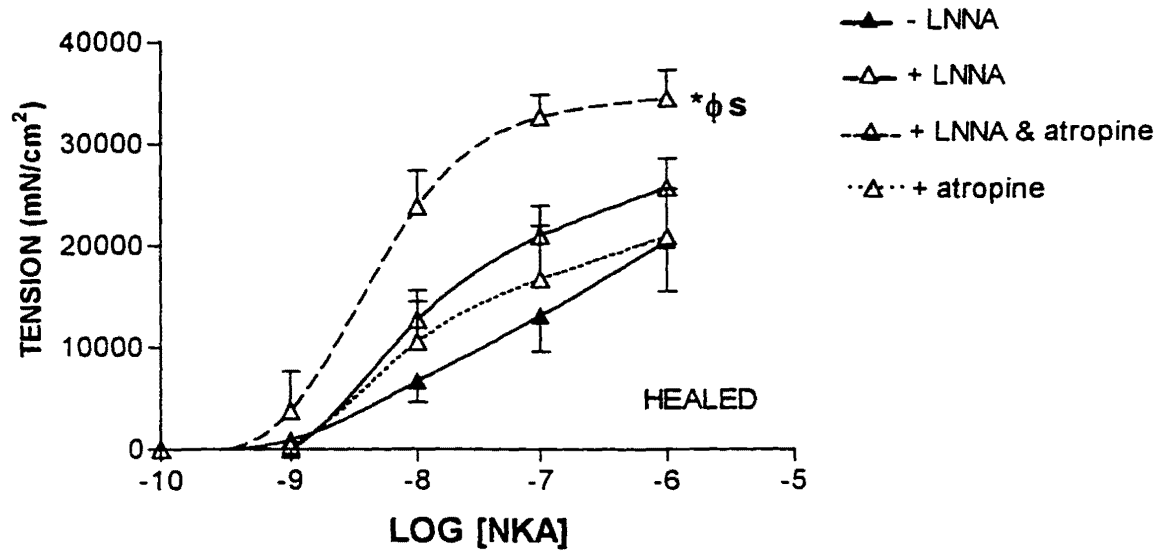
Based on studies using the NOS inhibitor L-NNA a return of nitrergic control was also seen in the healed tissue. Like the control muscle, in the presence of L-NNA, healed muscle showed an increase in response to SP, with no change in NKA response. In addition, healed tissue showed a similar contribution to the prefered antagonist AG, suggesting that iNOS had an inhibitory control of SP and NKA (Table 3 and Figure 16a and b). These results suggest a return for the role of NO in the inhibition of SP responses.

FIGURE 16

a.



b.



* $p < 0.05$ from - LNNA

^{s} $p < 0.05$ from + atropine

$^{\phi}$ $p < 0.05$ from + LNNA

FIGURE 16. SP (a) and NKA (b) concentration response curves for HEALED in the the presence and absence of L-NNA, atropine or L-NNA + atropine. Results are mean ± SEM.

In the presence of L-NNA and atropine, healed tissue also demonstrated an increase in response to both tachykinins, However unlike the response of control muscle to SP, responses in the presence of L-NNA and atropine were not significantly different from those in L-NNA or atropine alone. This suggested a loss of the additive effect seen in control tissue that was attributed to the independent effects of both muscarinic and nitric oxide mechanisms. In addition, NKA responses in L-NNA and atropine showed an additive effect that was not evident in control muscle. These results are shown in Table 3 and Figure 16a and b.

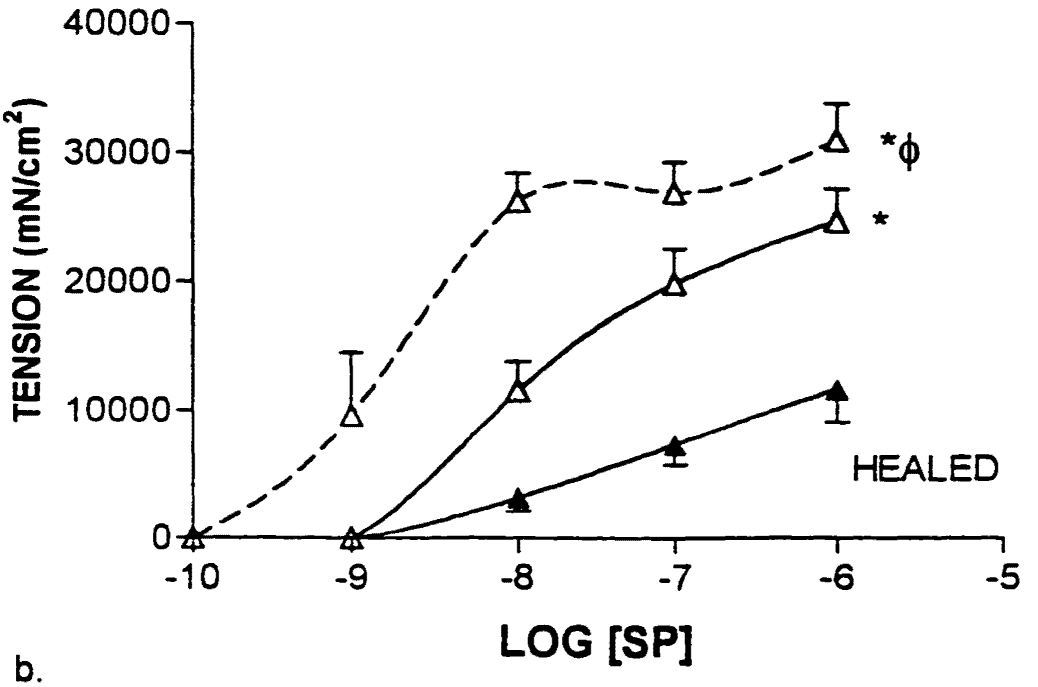
The contribution of other inhibitory mechanisms to the overall contractility of healed smooth muscles were found to be similar to that of control. For both tachykinins there was a significant increase in the presence of L-NNA and apamin that was greater than that in L-NNA alone. This suggested that healed tissue, like control, had a significant contribution of inhibitory neurotransmitters that utilized K^+ channels. resulting in hyperpolarization and consequent relaxation of smooth muscle. Results for this group of studies are shown in Table 3 and Figure 17a and b.

Responses of SP to TTX were similar to control, while NKA demonstrated no TTX sensitivity. In the presence of HEX, healed tissue showed an increase in response to SP. These results were similar to that of control demonstrating the return of the contribution of ganglionic and neural innervation to smooth muscle responses to SP. The results of additions of HEX or TTX to healed muscle strips are shown in Table 3, Figure I8a,b, c.

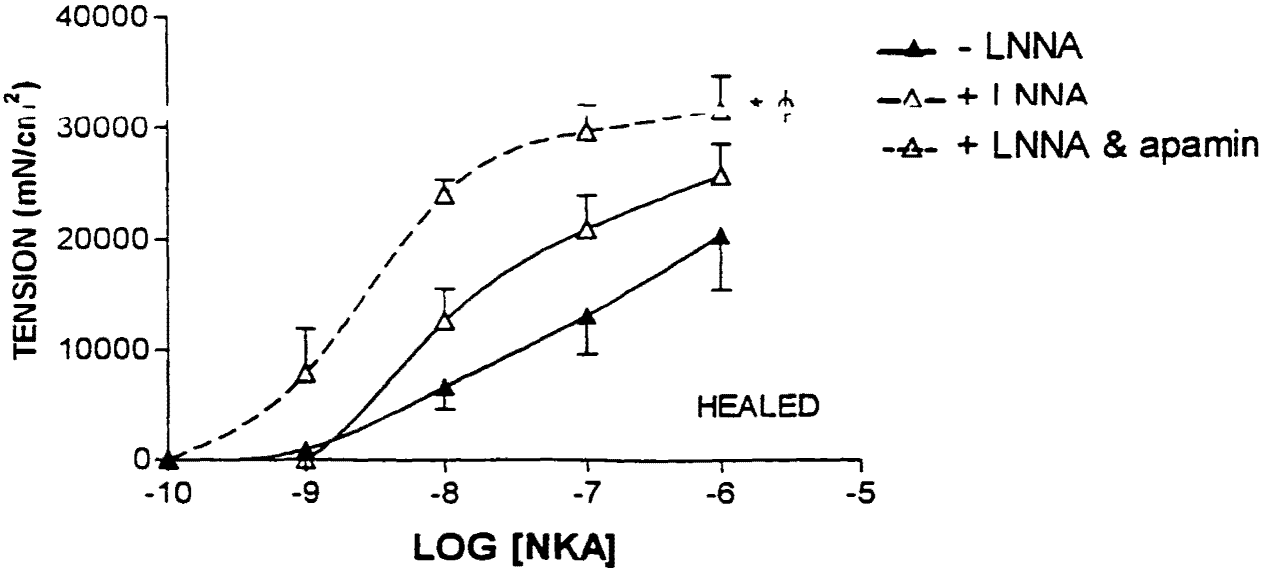
The responses for healed muscle mimicked that of control for SP. Healed tissue showed a return of responses for tachykinins to control, suggesting healing restored nitrergic, cholinergic and ganghonic influence.

FIGURE 17

a.



b.

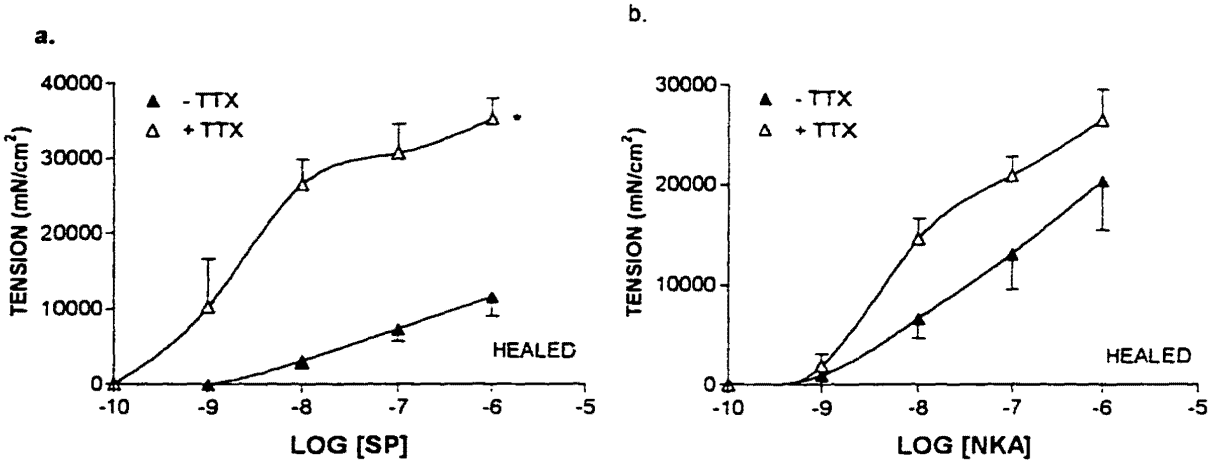


* $p < 0.05$ from - LNNA

ϕ $p < 0.05$ from + LNNA

FIGURE 17. SP (a) and NKA (b) concentration response curves for HEALED in the presence and absence of L-NNA or L-NNA + apamin. Results are mean + SEM.

FIGURE 18



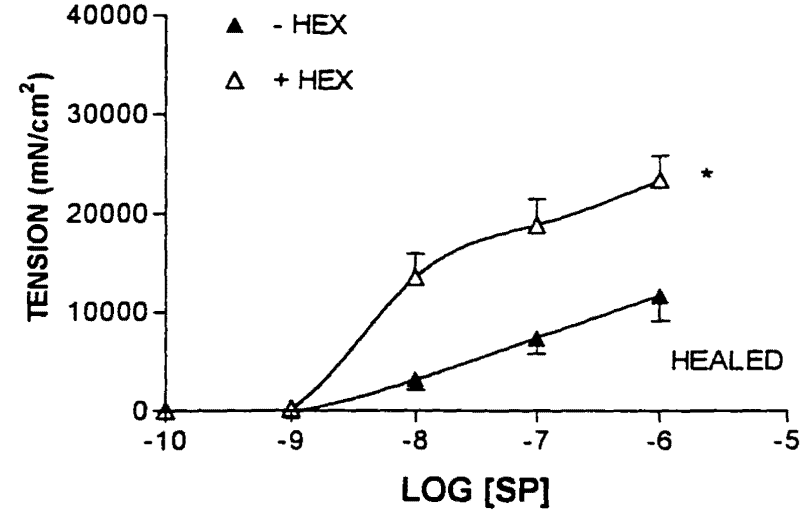


FIGURE 18. SP (a, c) and NKA (b) concentration response curves for HEALED in the presence of TTX (SP and NKA) and HEX (SP). Results are mean ± SEM.

REINFLAMED

In contrast to the changes observed in acute tissue when compared to control muscle, responses seen in the reinflamed tissue to tachykinins were no different from healed muscle. In the presence of atropine, reinflamed muscle demonstrated an increase in response to SP and no change in the NKA response. This suggests a possible remodeling of cholinergic pathways during reinflammation. Table 3 and Figure 19a, and b show the results of these studies.

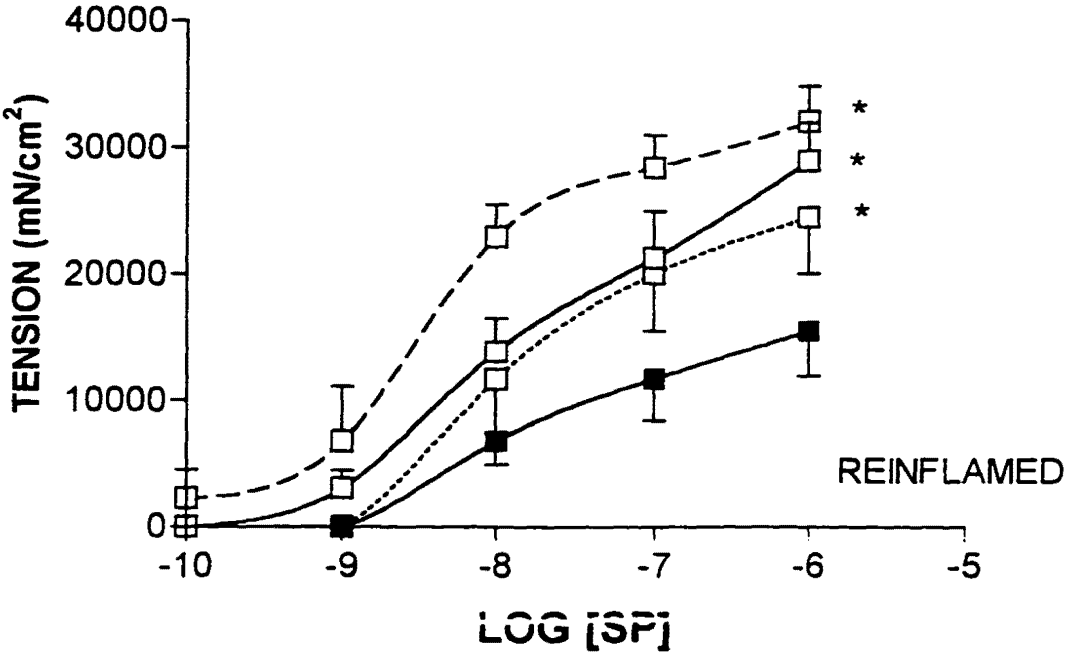
The response of reinflamed tissue to SP in the presence of L-NNA were similar to those seen in healed muscle, but decreased from that seen in the acutely inflamed group. In addition, in healed muscle only the responses to SP were increased in L-NNA where as in reinflamed muscles responses to both tachykinins were enhanced by L-NNA. Addition of aminoguanidine resulted in similar increases in tachykinin responses to those seen in healed, control and acutely inflamed tissue (Table 3).

Blocking the action of both NO and Ach resulted in significant increases in the responses of both tachykinins, similar to healed tissue, but unlike acutely inflamed tissue, there was no loss of cholinergic or nitrergic control (Table 3 and Figure 19a, b).

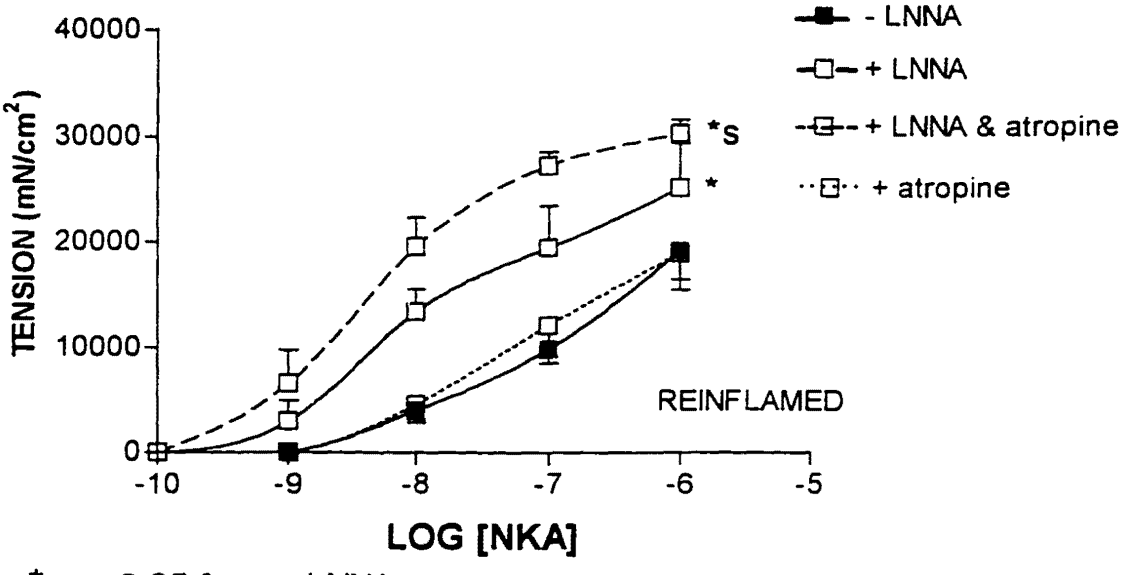
When the actions of all inhibitory signals were blocked to the smooth muscle (L-NNA + apamin) there were increases in response to both tachykinins. In addition, similar to healed tissue, there was a significant contribution of other inhibitory neurotransmitters to the response. As previously noted, these changes were not seen during the the initial acute inflammation. This suggests a change in the control of inhibitory mechanisms upon reinflammation. Results for responses to tachykinins in the presence of L-NNA and apamin are shown in Table 3 and Figure 20a and b.

FIGURE 19

a.



b.



* $p < 0.05$ from - LNNA

^{s} $p < 0.05$ from + atropine

FIGURE 19. SP (a) and NKA (b) concentration response curves for REINFLAMED in the presence and absence of L-NNA, atropine or L-NNA + atropine. Results are mean ± SEM.

FIGURE 20

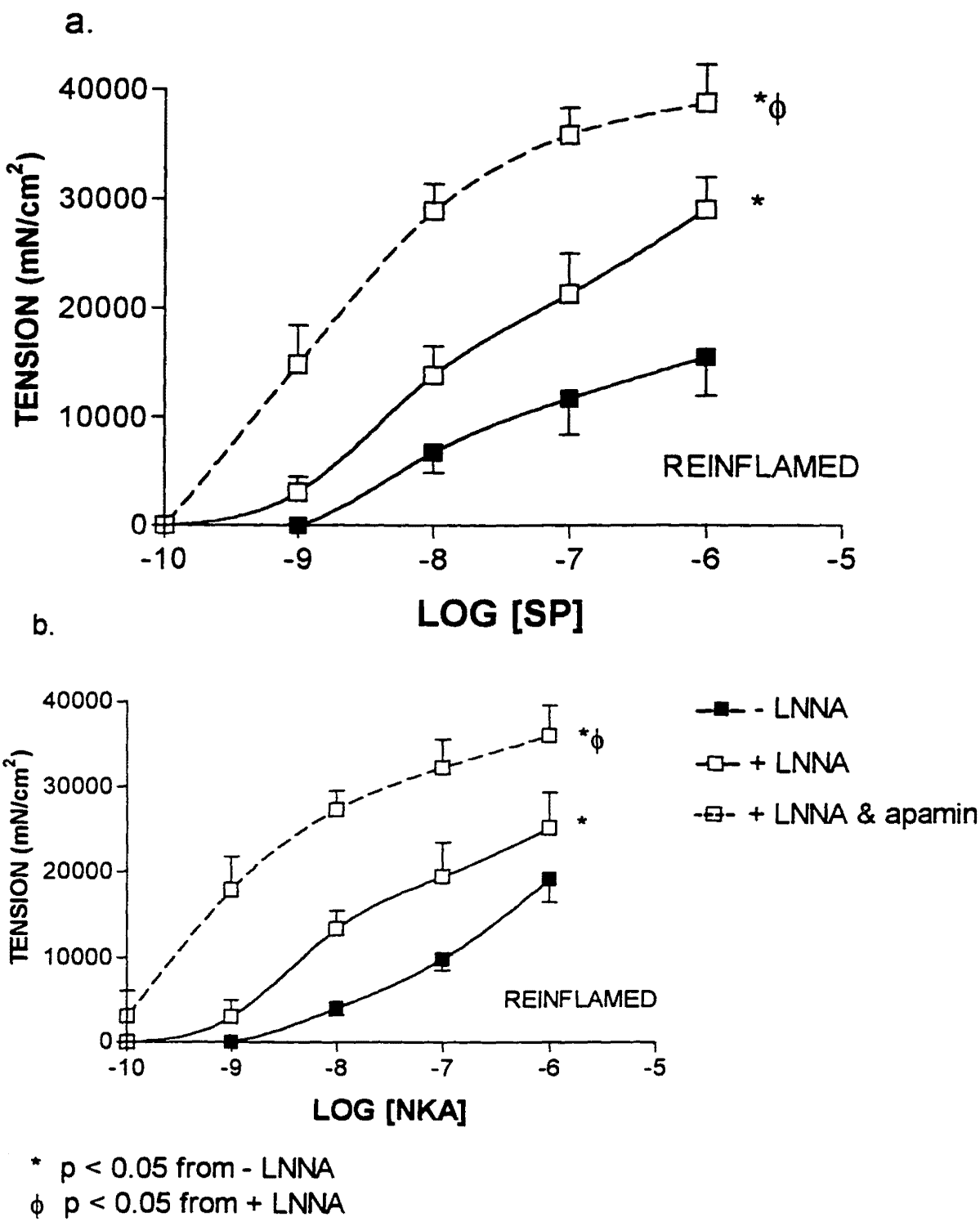


FIGURE 20. SP (a) and NKA (b) concentration response curves for REINFLAMED in the presence and absence of L-NNA or L-NNA + apamin.
Results are mean ± SEM.

Finally, reinflammation resulted in an increase in both tachykinin responses in TTX and to SP, in the presence of HEX. These results were no different from responses seen in healed tissue for SP again suggesting the role of neural and ganglionic conduction in the transmission of inhibitory signals (Table 3 and Figure 2la, b, and c). Similar to healed tissue, reinflammation resulted in significant increases in responses to the tachykinins from baseline values for many of the antagonists used. These increases were in contrast to the changes in tachykinin responses observed in the acute inflammation. This was particularly evident when studying the changes in cholinergic and nitrergic mechanisms after reinflammation of the healed area. Unlike acute tissue, reinflamed (and healed) tissue maintained cholinergic and nitrergic control for SP suggesting changes in the ultimate release of inhibitory neurotransmitters.

To summarize the changes seen in the responses to tachykinins, only that of neural conduction (TTX) and the suggested contribution of iNOS (AG) were preserved throughout all stages of inflammation. These cholinergic and ganglionic control mechanisms, as well as the contribution of NO (+ L-NNA), were found to be lost in acute tissue, regained during healing and maintained in reinflammation. In addition, except for acute tissue, other inhibitory neurotransmitters played an important role in the regulation of smooth muscle contractility. The return of overall inhibitory mechanisms may account for the apparent quiescence of clinical signs seen during healing, but at the expense of remodeling of cholinergic and nitrergic pathways that only become evident during subsequent reinflammation. With the loss of NOS staining there was likely a shift in the source of NO from that of cNOS to iNOS and depending on the number and activity of macrophages, this would explain the return of inhibitory NO control.

FIGURE 21

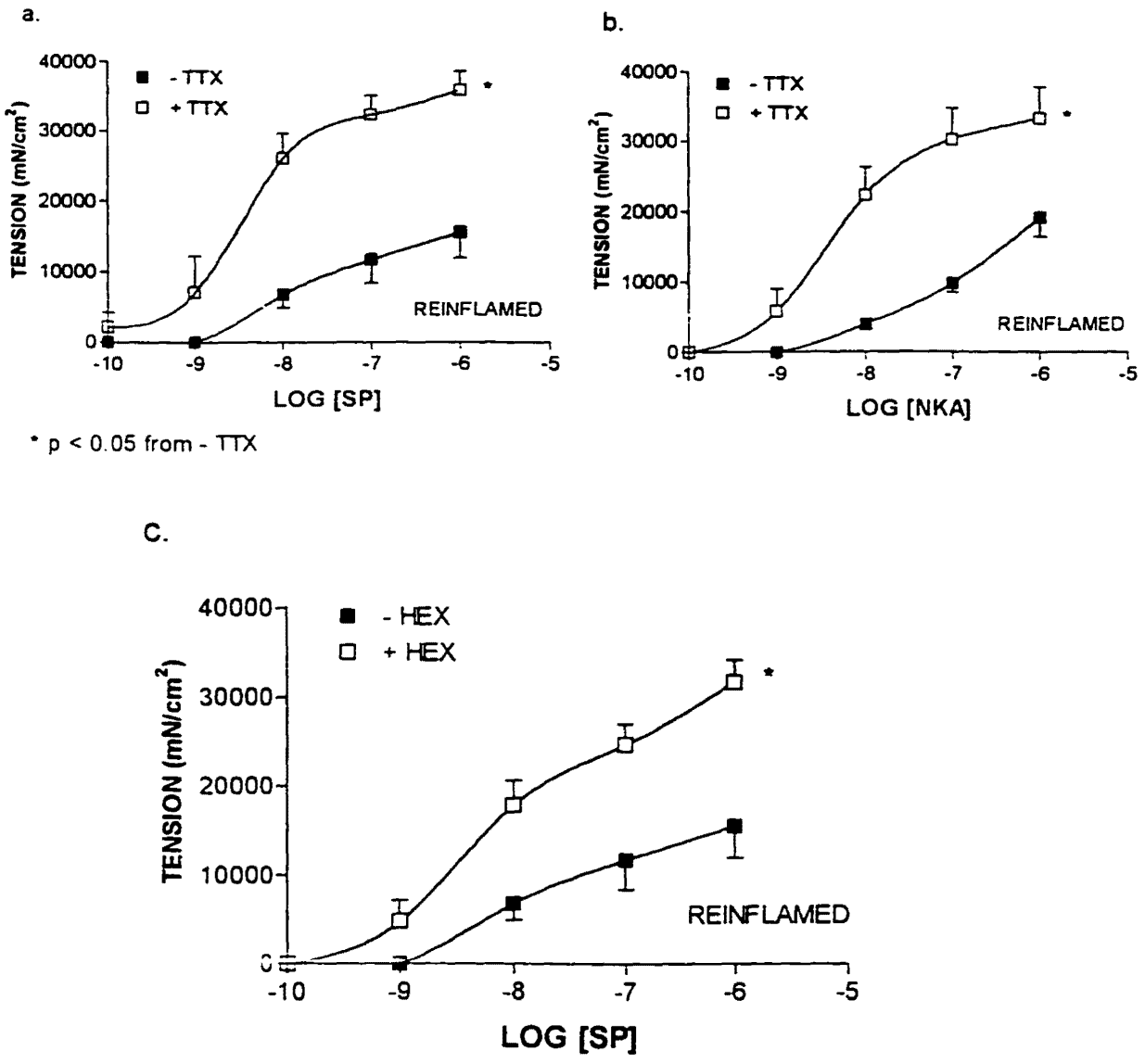


FIGURE 21. SP (a, c) and NKA (b) concentration response curves in REINFLAMED the presence of TTX (SP and NKA) and HEX (SP). Results are mean ± SEM.

DISCUSSION

These experiments demonstrate that during the course of inflammation there are alterations in both the inherent properties of smooth muscle as well as the neural input to smooth muscle. These changes were initiated in acute inflammation and appeared to have a lasting effect throughout the healed stage. Moreover, the response to reinflammation was significantly different from the acute inflammation. These changes were due to a combination of several factors including alterations in spontaneous contractions, changes in sensitivity to depolarization in smooth muscle, and remodeling of neural pathways.

The ability of the muscle to contract is a function of several critical physiological parameters including 1) resting membrane potential, 2) the regional basal electrical rhythm, 3) the ionic driving force changes, and 4) the normal morphology of smooth muscle cells. In the colon, the net input of excitatory and inhibitory neurotransmitters via the enteric nervous system influence and control colonic smooth muscle and determine normal motility patterns. An important feature of the neural control is a tonic inhibition of the inherently excitable smooth muscle syncytium. Spontaneous contractions reflect these influences and are changed through the course of inflammatory processes.

SPONTANEOUS CONTRACTIONS

The results of these studies support a role for NO in the generation of myogenic spontaneous contractions. Immunohistochemical localization of NOS in the rat colon are in agreement with that reported in Shuttleworth, et al. (1993) and Domoto, et al. (1995) in

dog colon suggesting that NO localized in nerves and neurons throughout the muscularis externa may serve as a neurotransmitter in the enteric ganglia. In the presence of the nonspecific NOS inhibitor, L-NNA, the amplitude and frequency of smooth muscle spontaneous contractions were increased in control muscle. This suggested that spontaneous contractile activity is tonically suppressed by basal release of NO. Similar results were also reported in canine (Keef, et al., 1997), and human colonic smooth muscle (Keef, et al. 1993) and rat intestinal smooth muscle (Calignano, et al., 1992) where increased muscle contractions were observed following administration of either L-NNA, or L-NAME.

There is evidence suggesting that spontaneous contractions are coupled to electrical slow waves and that the electrical and mechanical behavior of the muscle can be altered by basal release of NO (Keef, et al., 1997). The combined action of NO at the interstitial cells of Cajal (ICC) and release of NO from these cells, suppress the inherently excitable smooth muscle cells. These investigators suggested that the release of NO by the ICC is responsible for the rhythmical basal pattern of electrical and mechanical activity found in the canine colon (Keef, et al., 1997b).

Initial inflammation resulted in lasting alterations that may be due to a loss of NO influence on the control of these spontaneous contractions.This is supported by the loss of NOS staining and L-NNA data. Acute inflammation increased the amplitude of spontaneous contractions and the contractions were also no longer sensitive to L-NNA suggesting a loss of NO's control on the strength of contraction. This is also supported by the lack of NOS staining in the nerves in this tissue. Concurrently, there was an increase in the sensitivity (EC_{50}) of smooth muscle to KCl. Acute inflammation did not

change the magnitude of the response to KCl, but increased sensitivity to KC1, suggesting an increase in the excitability of smooth muscle. It seems unlikely that the contractile machinery is changed since the maximum response to KC1 and basal parameters of the muscle (active, total, passive tensions, weights and L_0) were unaltered from that of control.

The cause of this increase in muscle contractility may be attributed to inflammatory mediators, such as LTC/D_4 (Sjogren, et al., 1994), LTB_4 (Rachmilewitz, et al., 1989), PGE_2 (Sjogren, et al., 1994), and interleukin (Rachmilewitz, et al., 1989) that are elevated during enteric inflammation. Recent studies have implicated the mast cell mediator LTD_4 in the mediation of abnormal motility in response to acute ileitis (Sjogren, et al., 1994). LTD_4 increases smooth muscle contractility by increasing the sensitivity of nerves to stimulation, and these investigators concluded that LTD_4 mediates the increase of myoelectrical response during intestinal inflammation.

In the present studies, the amplitude of spontaneous contractions returned to control levels and regained control by NO in the healed tissue. The decreased sensitivity of the smooth muscle to depolarization (by KCl) may have also contributed to this return to control conditions. Subsequent acute reinflammation resulted in an increase in the amplitude of spontaneous contractions that like initial acute inflammation was not enhanced further by L-NNA. Thus, inflammation invariably produced a temporary increase in the strength of spontaneous contractions that may be in part due to a loss of inhibitory NO control.

In addition to changes in the amplitude of spontaneous contractions, the frequency of spontaneous contractions was also affected by inflammation. Frequency was increased in

healed tissue and remained elevated in reinflammation. This increase in L-NNA and/or AG sensitivity, was not evident in the acute muscle. In the presence of AG, frequency returned to control levels in both healed and reinflamed muscles. These data suggest that after the initial inflammation there is an increase in the frequency of smooth muscle spontaneous contractions that is controlled by iNOS activity. This is further supported by the absence of the constituitive neural NOS staining in these tissues.

The contribution of the inducible isoform of NO to the control of the frequency of spontaneous contractions during inflammation was unexpected. In the normal colon, it is suggested that the basal release of NO suppresses spontaneous electrical and contractile activity, causing a slow oscillatory pattern. This pattern is dependent on an intact myenteric region with its associated enteric neurons and ICC (Keef, et al., 1997). Recent studies have demonstrated that NO may have different effects on smooth muscle vs ICC. NO reduces intracellular Ca^{+2} in smooth muscle causing relaxation, but increases intracellular Ca^{+2} in isolated ICC. This effect on ICC causes these cells to increase their activity and release NO (Sanders, 1996; Publicover, et al., 1993). The elevation of suspected iNOS activity during inflammation (acute, healed and reinflammation), resulting in an enhanced production of NO, may have altered the activity of the ICC and therefore influenced the frequency of spontaneous contractions throughout inflammation. Although there is little or no information on alterations in spontaneous contractions during inflammation, a change in the relationship between ICC, smooth muscle cells and nerves would explain the increase in frequency observed in this study. Inflammatory mediators may also contribute to the effect on frequency of spontaneous contractions. These mediators may damage or sensitize pacemaker cells responsible for the generation and

control of spontaneous contractions (Giovanni, et al., 1997). Furthermore, they may be responsible in part for the increased iNOS activity.

In conclusion, the process of inflammation alters both smooth muscle contractility and NO control of both the amplitude and frequency of phasic smooth muscle contractions. This suggests alterations in both pacemaker and smooth muscle cells, resulting in changes in the responses to other neurotransmitters.

NEURAL CHANGES-EXCITATORY NEUROTRANSMITTERS

The results of these studies demonstrated the importance of the cholinergic neural pathways in the enteric nervous system on changes in smooth muscle contractility during inflammation. Circular smooth muscle from control animals exhibited concentration dependent increases in tension in response to SP, NKA, and Ach. All of these transmitters have been shown to act to depolarize smooth muscle resting membrane potential thereby enhancing slow wave plateau phase duration and amplitude (Huizinga, et al., 1984; Sarna, 1991; Galligan, 1993). There is much evidence implicating these excitatory peptides as important neurotransmitters in the enteric nervous system.

The response to SP and NKA in control smooth muscle were similar to those reported for these tachykinins in other studies (Fountaine and Lebrun, 1989; Koelbel, et al., 1989). Both SP and NKA are excitatory neurotransmitters and several studies have demonstrated their ability to depolarize smooth muscle (Koelbel, et al., 1989; Tsukamoto, et al., 1997; Galligan, 1993). SP and NKA act on smooth muscle by two pathways. One is direct through the NKl (SP) and NK2 (NKA) receptors on smooth muscle, and the other action is indirect via NKl and NK2 nerves. Although both NKA and SP act as

excitatory neurotransmitters, they may result in the release of excitatory or inhibitory neurotransmitters at the smooth muscle. The increased response of SP and NKA in the presence of the Na^+ channel blocker TTX, suggested that the tachykinins binding to NKl and NK2 receptors in nerves resulted in a release of an inhibitory neurotransmitter at the muscle. In the absence of this inhibitory neural influence, the direct excitatory action of SP and NKA on smooth muscle was observed. These results were also noted in other studies (Fontaine and Lebrun, 1989; Foxx-Orenstein and Grider, 1996).

These data also suggest that stimulation of receptors in the myenteric ganglia results in a net inhibition of smooth muscle. Blockade of myenteric ganglia with hexamethonium resulted in an increase in the response to SP, indicating that SP, acting via the myenteric plexus, also results in release of an inhibitory neurotransmitter at smooth muscle. Similar results were found by Koelbel, et al. (1989) Fontaine and Lebrun (1989) and Foxx-Orenstein and Grider (1996). Finally, in this study there was an increase in the response to SP in the presence of atropine suggesting that there is a muscarinic cholinergic control of SP.

In summary, control smooth muscle demonstrated a large degree of inhibitory influence through the action of SP via nerves. The role of neurokinin A's role is less of neural origin because its receptors are believed to be located primarily on smooth muscle rather than nerves (Galligan, 1993). Although NKA shows preferential specificity for NK2 receptors, on smooth muscle (Galligan, 1993), NKA cross reacts with NKl receptors (Galligan, 1993). However, responses to both SP and NKA were elevated in the presence of TTX indicating that both have effects on tachykinin receptors on enteric nerves. These receptors are likely to be NKl. This may account for some of the

similarities for the responses of NKA and SP. However, differences m the responses to SP and NKA in the presence of atropine were noted. While SP showed an increase in response in the presence of atropine, NKA showed no such sensitivity. These data suggest then that although changes occur at the muscarinic receptors for the two tachykinins, the pathways taken must differ, since the regulation of the atropine neural path are opposite for these tachykinins.

There were profound histological changes in the inflamed tissues in this study, which is consistent with studies in other inflamed animal models (Conner and Grisham, 1996; Rachmilewitz, et al., 1989; Percy, et al., 1993b) of ulcerative colitis and IBD patients (Janowitz and Mauer, 1991; Berkow 1994). Damage and inflammation in colonic tissue may alter the inherent contractile properties of smooth muscle as well as its response to enteric neurotransmitters. It has been shown that enteric neurons are modified with evidence of degeneration and subsequent proliferation that may be sufficient to change neurotransmitter release (Koch, et al., 1990, Stark and Szurszewski, 1992).

In contrast to control responses, acute inflammation resulted in a significant increase in the responses to Ach, SP, and a decrease in the response to NKA. During inflammation SP levels have been found to be increased (Goldin, et al., 1989; Koch, et al., 1987; Mantyh, et al., 1988; Hosseini, 1996) and NKl receptor numbers elevated (Mantyh, et al., 1988). SP has been suggested to increase the frequency of GMC's (giant migrating complex) during inflammation (Tsukamoto, et al., 1997). Responses to Ach were also found to be altered during inflammation of the small intestine or colon (Shi and Sarna, 1997; Collins, et al., 1989; Hosseini, 1996).

The increase in response to SP and the decrease seen for NKA could arguably be due

to an increase and decrease in sensitivity of the muscle to SP and NKA respectively. However, this seems unlikely as EC_{50} values were similar for both tachykinins and in both control and acute tissue. In addition, responses for SP and NKA were measured in the presence of TTX. These two findings imply intact nerves with little change in the tachykinin receptor sensitivity at the muscle. It seems more likely that a change in pathway patterns occured. This is supported by the observations of a decrease in inhibitory input via cholinergic and nicotinic receptors in the responses for SP and an aqcuisition of inhibitory control in the responses for NKA during acute inflammation.

The cause for these changes seen in inflammation could be due to different reasons. One hypothesis may be that inflammatory mediators sensitize nerves to the action of neurotransmitters resulting in a greater contractile state. Alternatively, a decrease in inhibitory influence on the smooth muscle would also result in an increase in contractility. This last hypothesis seems more plausible based on results seen in this study as outlined based on unaltered EC_{50} values for the tachykinins in inflammation and the loss of sensitivity to atropine and hexamethonium for SP in acute inflammation and the loss of NOS staining at acute inflammation.

While there are many studies demonstrating histological evidence of chronic inflammation in animals (Rachmilewitz, et al., 1989; Collins et al., 1996; Percy et al., 1993b; Yamada, et al,, 1993; Ribbons, et al., 1995) and IBD patients (Roediger, et al., 1986; Singer, et al,, 1996; Berkow, 1994; Janowitz and Mauer, 1991), there are few studies reporting smooth muscle contractility changes during healing or reinflammation. In this study, healed muscle showed a decrease in the sensitivity to KCl, yet the maximum response to KC1 remained the same. Qualitatively similar results were found for a 24 hour

post TNBS-induced colitis in rats by Mourelle, et al. (1996).

During healing, nearly all of the responses of the smooth muscle to Ach and tachykinins, in the presence and absence of different antagonists, were similar to vehicle control tissue. Moreover, reinflammation did not significantly change the concentration response curves to the excitatory neurotransmitters Ach, SP and NKA. This was in direct contrast to what was expected based on responses during acute inflammation. However, despite the similarity of the responses in control, healed and reinflamed tissue, cholinergic and nicotinic control of the responses to SP were different from those observed after acute inflammation. This suggests a remodeling of neural input to smooth muscle occured sometime during the healing process. The remodeling of neural pathways during this time is supported by several observations. First, during acute inflammation, there was a change in the cholinergic and ganglionic control for SP (loss) and NKA (gain) responses. Although these controls appeared to be present in healed tissues, they were clearly absent in reinflammation. Second, the increased response to NKA in acute inflammation and the acquisition of cholinergic control for this tachykinin were not maintained throughout the later stages of inflammation. The increase in frequency of spontaneous contractions during inflammation also suggests a change in neural signals to the muscle as early as the initial inflammation. Alternatively, there may have been a change in the amount or type of neurotransmitters released at the level of the smooth muscle.

The loss of neuronal NOS staining suggests a contribution of NO to muscle responsiveness. This may also have an effect on the changes in the response to SP and NKA during the course of inflammation that may involve adjustments in the NO inhibitory neural control (Boughton-Smith, et al., 1993; Mourelle, et al., 1996; Ribbons, et al., 1995;

Singer, et al., 1996). Inflammation mediated changes in the levels of NO may contribute to smooth muscle responses during the healed and reinflamed stages. It should be noted that in this study the effect of tachykinins on smooth muscle (in the presence of TTX) was conserved throughout all stages of inflammation. This is not inconsistent however, with a remodeling of intact neuronal pathways that involve NO.

NO CONTROL

Smooth muscle responses to the tachykinin in the presence of the NO antagonists in control muscle in this study demonstrated the importance of NO inhibition. NOS containing neurons have been located in nerve fibers in the myenteric ganglia (Shuttleworth, et al., 1993; Domoto, et al, 1995). NO release from enteric nerves has been demonstrated to function in regulating and controlling the excitability of the smooth muscle (Ward, et al., 1992; Delbro, 1996; Keef, et al., 1993). Morphological studies of canine colon suggest that nitric oxide is also a critical intermediary in the communication between Interstitial cells of Cajal (ICC), enteric inhibitory nerves, and smooth muscle (Keef et al, 1997; Xue, et al., 1994). Shuttleworth, et al. (1993) found an increase cGMP level in the ICC suggesting that NO may have an important influence on eliciting a post-junctional response in these pacemaker cells. All these sources of NO contribute to the overall contractility of the smooth muscle. In addition, evidence for NO release via iNOS from macrophages (Marletta, et al., 1988; Moncada, et al., 1991) and intestinal smooth muscle (Grider, et al., 1992; Nichols, et al., 1994) and vascular endothelial cells (Furghott and Zawadzki, 1980) provide further support for NO's role in the GI tract. Resident colonic bacteria and their products have also been demonstrated to have an important role

in the induction of the iNOS and therefore NO release from macrophages (Mourelle, et al., 1996). This would include both macrophages normally present in the colon, and additional macrophages that migrate into the colon as part of the inflammatory response.

The present studies suggest that there is a neural release of NO by excitatory tachykinins. The increased response to SP in the presence of LNNA, under control conditions supports this conclusion. The increase in response of SP in the presence of atropine suggest that SP releases an inhibitory neurotransmitter via cholinergic pathway and the increase response of SP in the presence of L-NNA suggest that this inhibitory neurotransmitter is NO. NKA appears to have neither of these controls.

As stated earlier, SP has receptor sites at both nerve and muscle (Galligan, 1993), however its major effect is within nerves to release both excitatory and inhibitory neurotransmitters such as NO. In the presence of L-NNA plus atropine, the response to SP was greater than that in the presence of either antagonist alone. This demonstrated that there are two separate pathways for the SP mediated release of an inhibitory neurotransmitter, one for NO, and a second for a nonnitrergic inhibitory neurotransmitter such as ATP or VIP. An increased response to NKA was noted only in the presence of both L-NNA plus atropine. Although NKA has receptors on both nerves and smooth muscle, the majority of its receptors are on smooth muscle (Galligan, 1993). This suggests that NKA may also act via NKl receptors on nerves to release an inhibitory neurotransmitter, but only when cholinergic and nitrergic pathways are stimulated concurrently. It should be noted that these data do not exclude the possiblity that Ach may also stimulate release of NO from enteric nerves by a pathway that does not involve either tachykinin. In the guinea pig ileum, Wiklund, et al. (1993) demonstrated that Ach

stimulates the release of NO via muscarinic receptors. However it would seem this effect does not contribute to the action of SP or NKA on NO release in the present experiments.

NO is also known to be produced by activated macrophages and other inflammatory cells via iNOS (Lefer and Lefer, 1993; Moncada, et al., 1991). In this study there was an increase in the response to both SP and NKA in controls in the presence of the prefered iNOS inhibitor, AG, suggesting that inducible NO controls smooth muscle response to the tachykinins even in the absence of inflammation. This contribution of iNOS was also noted throughout the different inflammation stages, suggesting that even during inflammation and healing, iNOS activity continues to contribute to muscle relaxation.

Although NO has a major role, evidence that other inhibitory neurotransmitters control smooth muscle responses (Grider, 1993; Makhlouf and Grider, 1993) cannot be overlooked. The influence of other inhibitory neurotransmitters utilizing potassium channels was demonstrated in control tissue based on the significant increase in response to SP and NKA in the presence of apamin. Neurotransmitters such as ATP, and/or VIP and NO have been found to be colocalized in neurons of the enteric nervous system (Matini, et al., 1995; Burnstock, 1994; Berezin, et al., 1994). The relationship of these inhibitory neurotransmitters VIP, ATP, and NO is a subject of great debate (Grider, 1993; Grider, et al., 1992; Maklouf and Grider, 1993; Huizinga, et al., 1991; Keef et al., 1993a). There are those who believe (Grider, 1993; Grider, et al., 1992) that NO produced from neurally induced relaxation is derived from muscle cells due to the actions of VIP, while others maintain (Huizinga, et al., 1991) that NO is involved more as an intermediary. The large effect of L-NNA and apamin on contractility in this study support the idea that in addition to NO, other neurotransmitters, utilizing potassium channel

mechanisms, are significantly involved in the inhibitory control of smooth muscle. Control studies reported here gave an insight into some of the neural pathways and mechanisms of actions used by these neurotransmitters. Figure 22 shows a possible pathway used by these neurotransmitters based on antagonist studies utilized here under healthy (control) conditions.

NITRIC OXIDE CHANGES IN INFLAMMATION

While control tissue demonstrated a significant nitrergic control on smooth muscle contractility, acute inflammation appeared to cause a loss of this control. There also appeared to be a remodeling of the nitrergic pathways similar to the changes seen in the cholinergic, and ganglionic pathways for acute inflammation. This is supported by the observation that the action of NO was also lost. The loss of muscarinic, ganglionic and nitrergic control all appeared to contribute to the excited state of the muscle in acute inflammation implying a loss of a portion of the inhibitory control. It is important to note, however, that there was a maintenance of a general neural (TTX) and iNOS (AG) control. This supports the conclusion that inflammation induced a remodeling of the nerves as opposed to a loss of neural pathways in general.

Although there was no increase in the response to tachykinin in the presence of L-NNA in acute colitis, there may have been an influence of NO from iNOS activity, particularly in the response to SP. Many studies have suggested a contribution of this isoform of NO from different sources (Boughton-Smith, et al., 1993; Mourelle, et al., 1996; Ribbons, et al., 1995; Singer, et al., 1996). The significant difference between the

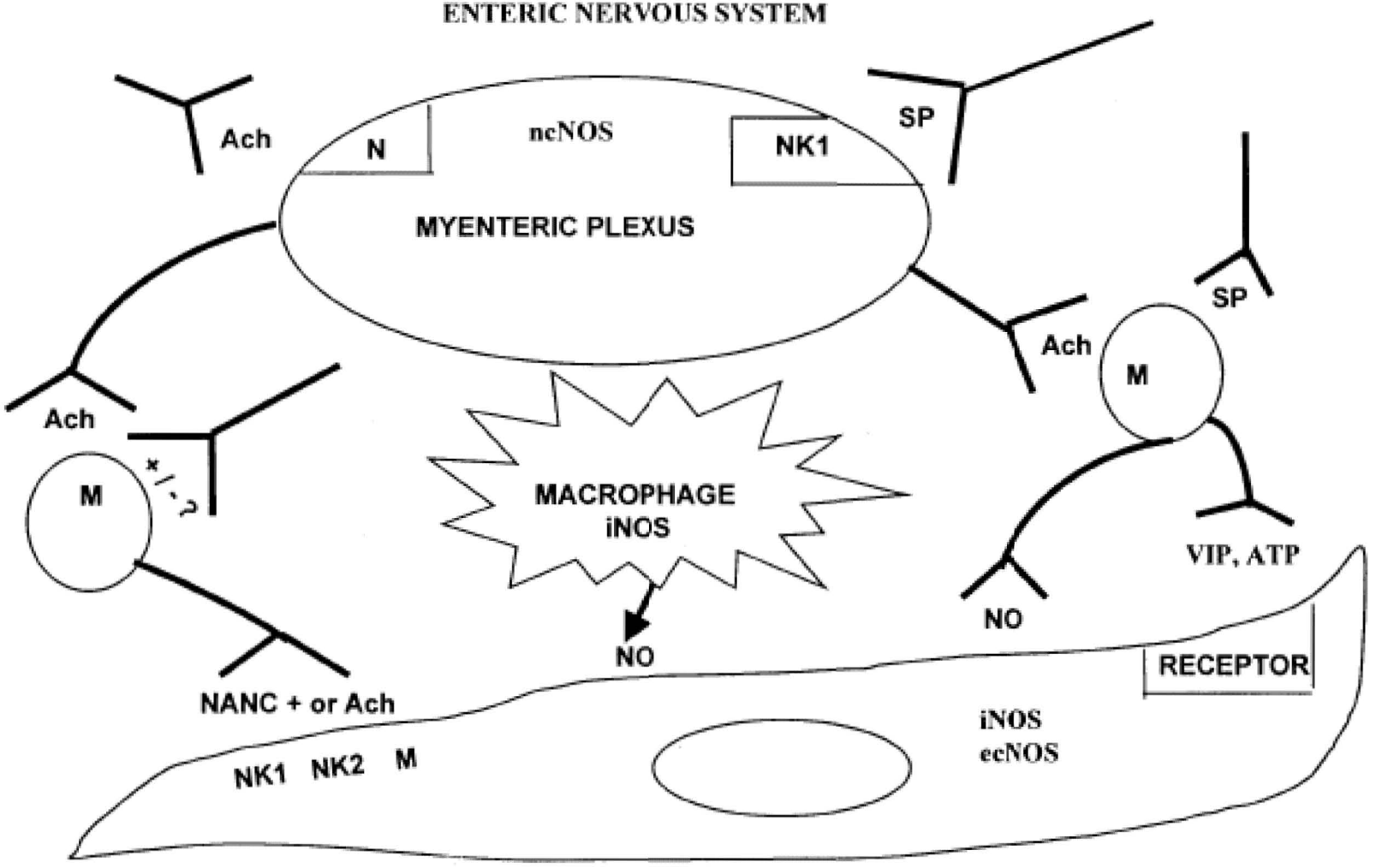


FIGURE 22: Proposed mechanism for neural pathways of the Enteric Nervous System in healthy (CONTROL) tissue.

response to SP in the presence of aminoguanidine versus that with L-NNA, suggests a greater contribution of NO from sources like inflammatory cells, such as PMN's and/or macrophages to the response. This is consistent with the reduction in neuronal NOS activity in this study, and emphasizes the importance of a contribution of NO from other sources such as epithelial, smooth muscle and/or inflammatory/immune cells to the control of smooth muscle excitability.

The excess NO produced during acute inflammation may also have contributed to the damage and/or alteration of neuronal pathways or sensitivity of smooth muscle to tachykinins. This together with an increase in sensitivity of acute muscle to depolarization would account for the increase response to SP and Ach. Excess NO resulting from iNOS activity has been suggested by several investigators to cause significant changes that include increases in vascular permeability (Boughton-Smith, et al., 1993) and increases in smooth muscle contractility (Mourelle, et al., 1996) via the formation of secondary reactive nitrogen and oxygen intermediates such as peroxynitrites, nitrosothiols, and transitional metals (Roediger, et al,, 1986; Lundberg, et al., 1994; Ribbons, et al., 1995). Observations by these investigators suggest a change in the chemical environment around enteric nerves and smooth muscles that would impact colonic motility.

Histological changes seen throughout inflammation in this study are evident in acute inflammation with a reduction of neuronal NOS (loss of staining) and significant increases in PMN's (acute) or agranulocytic type infiltrations (healed and reinflamed). Inflammatory cells are linked to excess NO production via iNOS (Marletta and Maxey, 1995; Moncada, et al., 1991) increased cytokine production (Rachmilewitz, et al, 1989), and/or elevated LTD_4 (Shea-Donahue, et al., 1997) all of which can potentially alter the

internal mileu of the colon. These secondary products of inflammation are likely to contribute to the remodeling of enteric neural control of smooth muscle by increasing sensitivity of nerves to stimulation as suggested previously (Shea-Donohue, et al., 1997). This increase in sensitivity of afferent nerves to stimulation would explain the hypersensitivity to stimuli seen in many of the symptoms of IBD.

In the healed model of inflammation nitrergic control (+ L-NNA) during responses to tachykinins also did not appear to be altered from control tissue. Nitrergic control (+ L-NNA), was temporarily lost during the initial inflammation but returned to control levels. Similar results for a 21 day post TNBS induced colitis in rats were found in Mourelle, et al. (1996). These investigators reported no changes in cNOS Ca-dependent activity at this time, however, they did report an increase in Ca-independent inducible NOS activity.

In the reinflamed model utilized in this study, NO antagonists resulted in responses to tachykinins that was significantly different from the acute muscle. In addition, neuronal NOS activity remained low in reinflamed tissue and was similar to that seen in acute and healed tissue. If there was a loss of neuronal NOS activity during acute inflammation then the apparent return of nitrergic control of smooth muscle responses to SP seen during the healed and reinflamed state are likely the result of NO from other sources such as macrophages, and inflammatory cells. An increase in NO from iNOS may explain in part the return of tachykinin and Ach responses to control values for healed tissue, and the lack of a response seen in reinflamed tissue. The effects of the excess NO generated from non-neuronal sources first emerged during healing but did not become significantly evident until reinflammation when differences were noted between reinflamed and acute tissue.

The data from the L-NNA plus apamin experiments also demonstrated that the

contribution of other inhibitory neurotransmitters was altered during inflammation. This became even more evident throughout the different stages of inflammation. During acute inflammation there was a loss of this neural influence much like that demonstrated for the cholinergic, nicotinic, and ganglionic pathways. However, in healed and reinflamed tissue this influence was regained, implying a return to normal. Remodeling and a sensitizing of nerves by increased levels of inflammatory mediators to release more SP and/or increased NO (iNOS) would likely account for this return, as proposed earlier. This would further support the role of iNOS increase during inflammation. If VIP and/or ATP neurons influenced the release of NO from muscle cells as proposed by Grider, (1993) and Grider, et al. (1992) a remodeling of these pathways could also explain the return to control levels in response to tachykinins during more chronic inflammation. This would support the idea that neurons are more likely remodeled due to a change in the environment during healing. The results seen between acute and reinflamed stages for the influence of these other inhibitory neurotransmitters further supports the hypothesis that there is a remodeling of these neural pathways in addition to other pathways discussed previously. In this study it seems very likely that increased iNOS activity, and inflammatory mediators all contribute to the return to control conditions for the responses to tachykinins in the healed and reinflamed.

Figure 23 and 24 show the proposed change in neural pathways and NO control for the acute (Figure 23) and the healed and reinflamed (Figure 24) states.

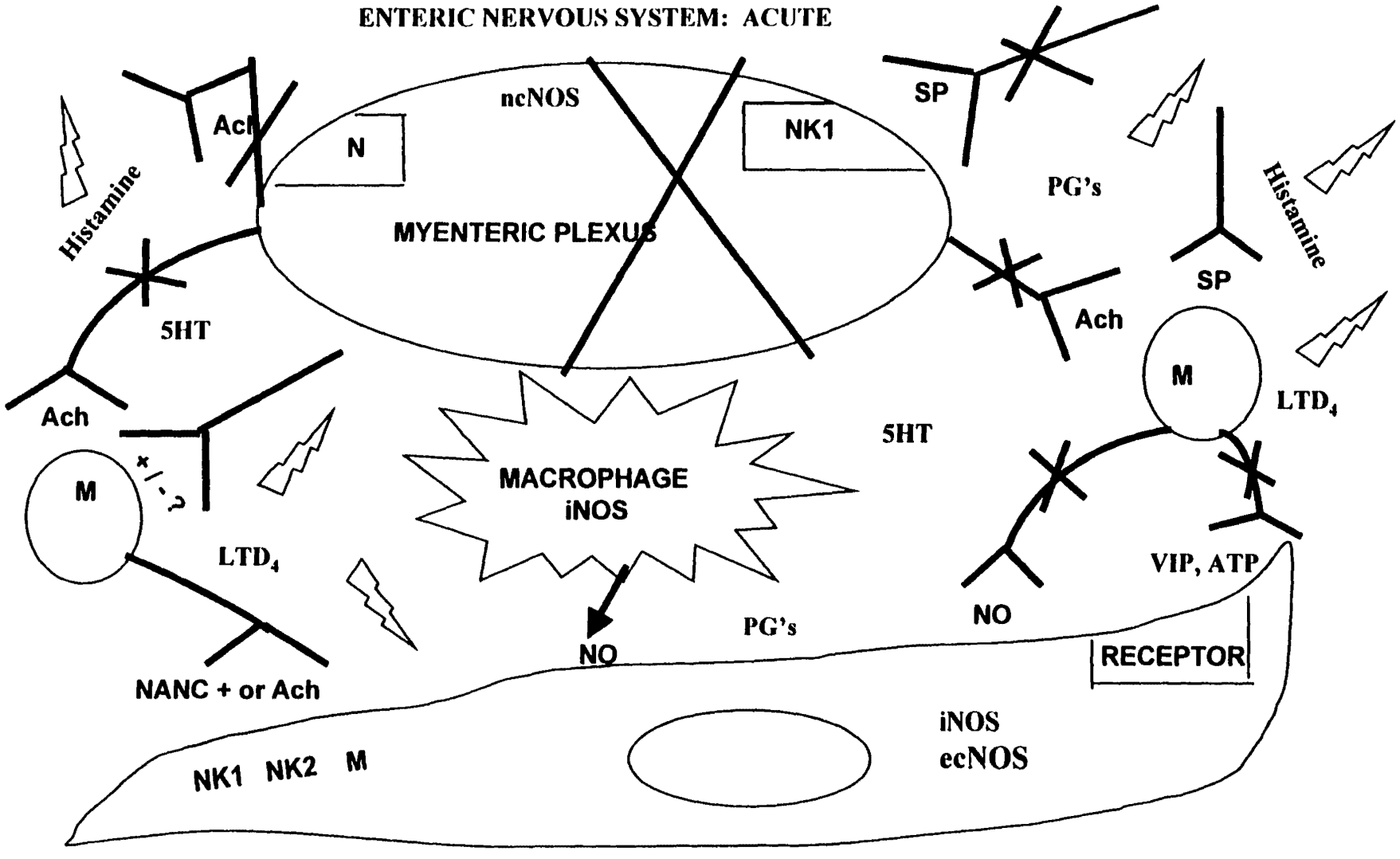


FIGURE 23: Proposed mechanism for neural pathways of the Enteric Nervous System in the acutely inflamed tissue.

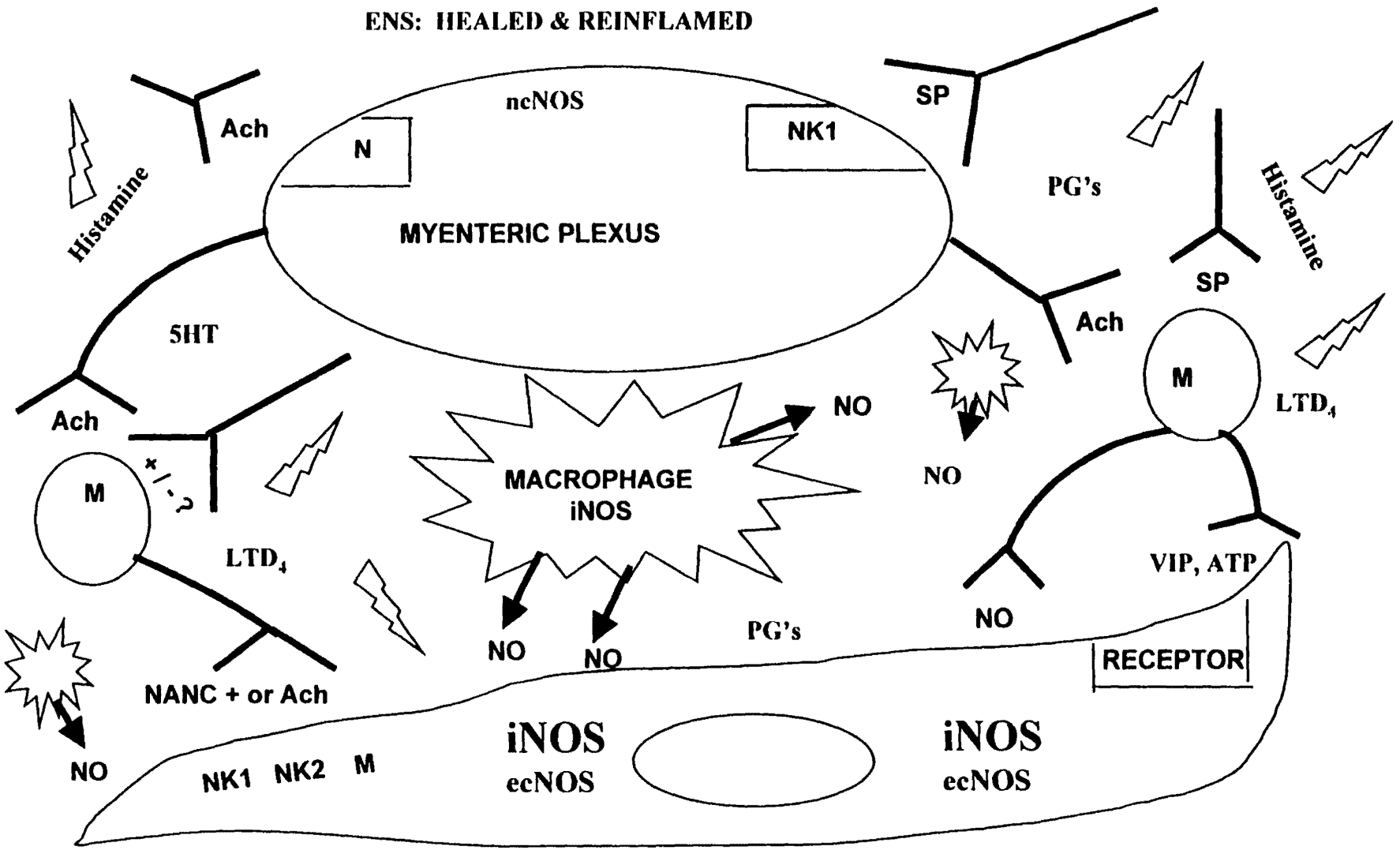


FIGURE 24: Proposed mechanism for neural pathways of the Enteric Nervous System in the inflamed tissue.

CONCLUSION

Crohn's disease and ulcerative colitis are chronic diseases, collectively termed IBD, that progress from an acute to a chronic stage of inflammation. They are further characterized by periods of quiescence during which spontaneous relapse or reinflammation occur. It is well recognized that IBD is associated with abnormal motility with diarrhea as the major clinical problem during the early stages interspersed with constipation and cramping with the increase in chronicity. The proposed studies were designed to investigate changes in the neural control of smooth muscle contractility during the course of inflammation. These data demonstrated that inflammation altered not only physical features of the distal colonic smooth muscle but also the excitability and contractile properties of the muscle. The initial inflammation induced a remodeling of neural control of the responses to the tachykinins. Although apparently masked during healing these changes increase expression at the time of reinflammation. This remodeling involved the initial loss of muscarinic, ganglionic and nitrergic control as well as changes in other inhibitory neurotransmitter pathways. Thus, changes in neural control of smooth muscle, the amount and type of inflammatory mediators, and smooth muscle sensitivity to depolarization all appear to contribute to the excited state of the muscle.

During inflammation there appears to be a shift of the source of NO control for inhibition from that of neuronal NOS to that of iNOS during inflammation.
It is the influence of NO generated from inflammatory cells and possibly other cellular sources that may maintain contractility of the smooth muscle more like control muscle.

However, this is without significant alterations of the enteric nervous system pathways and changes in spontaneous contractions of the smooth muscle. During the subsequent periods of reinflammation this remodeling may contribute to the abnormal motility patterns characteristic of IBD.

In this study the remodeling of the neural pathways and change in the contribution of NO that occurred during healing resulted in an apparent return to control. These changes seen in our model at healing may likely explain the apparent return to normal motility patterns clinically seen in the quiescent state of IBD. However, like chronic cases of IBD our model showed histological evidence and characteristics of motility changes upon reinflammation. These included alterations in spontaneous contractions that may likely contribute to diarrhea and cramping during clinical relapses IBD patients.

The morphological appearance of the colon in our model is similar to that of patients with persistent ulcerative colitis. In this study, reinflammation results in a remodeling of neural pathways and a change in the nitrergic control of smooth muscle. This remodeling is initiated during acute inflammation. In healed muscle there was an apparent return to normal motility patterns. This process mimics the quiescent stage in IBD. Changes that are present during reinflammation that may contribute to the abnormal motility observed in IBD are the increase in the frequency of spontaneous contractions and the increase in the response to a depolarizing stimulus (KCl). In addition there appears to be a loss of nitrergic neural control in regulation of smooth muscle contractions with an acquisition of control via iNOS.

APPENDIX

Table 4. Enteric Nervous System neurotransmitters studied and their receptors, mechanisms of action and antagonists.

Neurotransmitter	Receptor	Mechanism of Action	Antagonist
Ach	Nicotinic (preganglionic) Muscarinic (postganglionic)	G protein receptors mobilizing intracellular [Ca^{+2}]	1. Hexamethonium (preganglionic) 2. atropine (postganglionic)
SP	NANC-NK1 (nerves, muscles)	Receptor operated Ca^{+2} channel	GR-82334
NKA	NANC-NK2 (primarily muscle)	increase $[Ca^{+2}]_E$ Influx	MEN 10,376
NO	NANC-activation of soluble guanylate cyclase	Activation of cGMP operated Ca^{+2} dependent K channel hyperpolarization	L-arginine analogs competitive NOS inhibitors 1. L-NNA for cNOS 2. AG for iNOS 3. Apamin-partial blocker
ATP	NANC-activation of soluble adenylate cyclase	Activation of cAMP operated Ca^{+2} dependent K channel hyperpolarization	apamin-K channel blocker
VIP			

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